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SEXUAL SELECTION IN A LIVEBEARING FISH WITH A COERCIVE MATING SYSTEM (WESTERN MOSQUITOFISH, *GAMBUSIA AFFINIS*)

A DISSERTATION

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By

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SEXUAL SELECTION IN A LIVEBEARING FISH WITH A COERCIVE MATING SYSTEM (WESTERN MOSQUITOFISH, *GAMBUSIA AFFINIS*)

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF ZOOLOGY

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This dissertation is dedicated to Dr. Marsh-Matthews, my mentor, colleague, and friend.

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PREFACE

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Chapter 1: Ethology

Chapter 2: Journal of Evolutionary Biology

Chapter 3: Ecology of Freshwater Fish

Chapter 4: Behaviour

CHAPTER I

Factors influencing male mating behavior in a species with a coercive mating system (western mosquitofish, *Gambusia affinis*)

Raelynn Deaton

Abstract

The livebearing fishes of the genus *Gambusia* are quickly becoming a model system to test alternative mechanisms of sexual selection in the form of coercive mating. I investigated effects of male and female body size, and correlated characteristics on male mating behavior in the western mosquitofish, *G. affinis*. Because larger females typically have larger broods in *Gambusia*, I predicted that males would attempt more copulations with larger females. Two-way ANOVA showed that female body size was a significant predictor of male mating behavior but male size was not. I also tested the effects of a suite of additional traits (both male and female) on male mating attempts. In a stepwise multiple regression, female size (SL), size of the gravid spot, and male testes mass were significant predictors of male mating attempts, accounting for about 27% of variation in male mating. Path analysis showed that differences between male and female body size, male body condition, and male testes mass were significant predictors of male mating attempts, and also accounted for 27% of the variation in male mating attempts. The two statistical models were very similar in their predictive power, but differed slightly in significant predictor variables. My results confirm that factors other than female size are important predictors of male mating behavior in the western mosquitofish.

Introduction

Since Darwin (1871), studies on sexual selection have focused primarily on two mechanisms: intersexual mate choice (female choice) and intrasexual competition (male-male competition; Andersson 1994). It is clear, however, that other strategies, such as sexual coercion (forced copulations), play important roles in the evolution of mating systems (Clutton-Brock & Parker 1995). Consequently, coercive mating has been proposed as a third mechanism of sexual selection (Andersson 1994) and likely drives the evolution of male traits in many organisms (Clutton-Brock & Parker 1995). It is the primary mating tactic in many animals (Clutton-Brock & Parker 1995), including monarch butterflies (Solensky 2004), water striders (Arnqvist & Rowe 1995), garter snakes (Shine & Mason 2005), bush crickets (Vahed 2002), and macaques (Cooper & Bernstein 2000). Coercive mating often is used as an alternative strategy (Gross 1996) in organisms with pronounced male size polymorphism (Zimmerer & Kallman 1989). In the guppy, for example, larger, more colorful males court females, while smaller males use sneaky or coercive behavior to obtain mating success (Houde 1997).

Poeciliids in the genus *Gambusia* (mosquitofishes) provide researchers with an excellent alternative system for studies on mate choice when traditional mechanisms of sexual selection (e.g. female choice, male-male competition) are weak or lacking (Bisazza et al. 2001). The *Gambusia* mating system is largely male driven; males of all sizes force females to copulate (Bisazza et al. 2000). Thus, male size (and other positively associated traits) is likely to be important in male reproductive success. In many livebearers, larger males are often more aggressive, and potentially better competitors for mates (Hughes 1985; Riesch et al. 2006). Bisazza et al. (2000) also

demonstrated that larger males prefer to defend larger females, forcing small males to interact more with smaller, less fecund females. However, Bisazza and Pilastro (1997) showed a small male mating advantage in the eastern mosquitofish, *G. holbrooki* (small males mated at higher rates than large males in a non-competitive situation), and suggested this as a potential mechanism for the coexistence of small and large males in natural populations. Hughes (1985) showed that small and large male *G. affinis* differ in mating behaviors, where small males forced-copulated (coerced) at higher rates than large males, suggesting size-correlated mating differences. However, this was true only when males were mated with sexually receptive females (Hughes 1985). Further, small males of the one-sided livebearer (*Jenynsia multidentata*) avoided mating with very large females, presumably as a predatory defense (Bisazza et al. 2000). Thus, there appears to be marked differences in mating behaviors between large and small males in many livebearers, possibly due to competition, body size, predator avoidance, and female receptivity.

In my study population, mature male western mosquitofish (*G. affinis*) range from about 11-27 mm standard length (SL) and, like most mosquitofishes, grow very little after sexual maturity (see Snelson 1989; Deaton own data). Little is known about the genetic basis of male size in mosquitofish, but in other livebearers (*Xiphophorus*), male body size has been linked to variation in the pituitary locus on the Y-chromosome (commonly referred to as the "P gene"; Zimmerer and Kallman 1989). However, male size in mosquitofish is also under some level of social control (Campton 1988), indicating environmental determinants of male size. Thus, understanding factors that maintain male

size variation in natural populations is of interest to those studying livebearing fishes, especially fishes of the genus *Gambusia*.

Female size can also be an important determinant of male mating success. In *G. holbrooki*, males prefer to mate with larger females (Bisazza et al. 2000), which is expected when larger females have larger broods (e.g. Marsh-Matthews et al. 2005). Although it also has been suggested that overt female choice is relatively unimportant in mosquitofishes (Bisazza et al. 1989, Bisazza and Marin 1991, 1995, & Bisazza et al. 2000, but see Hughes 1985, 1986; McPeek 1992; Gould et al. 1999; Bisazza & Pilastro 2001, & Langerhans et al. 2005), recent studies suggest that females may have more control over male mating than was previously thought (Bisazza et al. 2001).

Factors other than body size also are known to affect the mating behavior of males in many species, including age (Savalli & Fox 1999), social dominance (Haley et al. 1994), and condition (Kissner et al. 2005). In many species, traits that affect reproductive success are strongly correlated with size, and therefore, may have indirect effects on male mating success (Wikelski 2005). In fishes, several male and female characteristics have been shown to affect male mating behavior and/or reproductive success, including territory size and/or quality (Kraak & Weissing 1996), pheromones (Park & Propper 2002), body pigmentation (Amundsen & Forsgran 2003), mating history (Dosen & Montgomerie 2004), male dominance/aggression (Gozlan et al. 2003), body condition (Kodrick-Brown 1985, 1989) and female reproductive state (Bisazza et al. 2000), many of which are correlated with body size.

I examined effects of female and male body size on male mating behavior in the western mosquitofish, *G. affinis*. I predicted that males would prefer larger females

because they are more fecund (Pelabon et al. 2003). Using a two-way ANOVA, I tested for size-correlated mating differences among males, which has been suggested for *G. affinis* (Hughes 1985). I predicted that males of all sizes would prefer to mate with larger, more fecund females. However, small males may avoid mating with much larger females in order to avoid predation. Finally, using two different statistical approaches, I tested the effects of a suite of correlated characteristics (both male and female) on male mating to determine important predictors (in addition to body size) of male mating behavior in the western mosquitofish.

Materials and Methods

In June 2005, I collected fish from a small pond in Norman, Oklahoma and returned them to a greenhouse laboratory. Fish were held in two 370-l community tanks and fed commercial flake food daily for two weeks. Since females were field collected, they were exposed to males previous to the experiment. In addition, because of difficulty collecting small males from the field, I reared male offspring from pregnant females collected from the same pond in May 2005. One week prior to behavioral tests, I visually separated males and females into three size classes (small, medium, and large). I included a medium male size class in the experiment to include all natural size variation. However, I did not make any *a priori* predictions regarding mating behavior of medium-sized males.

The size classes included little to no overlap, with average SL for small, medium, and large males 15.4$\pm$1.03 mm, 18.4$\pm$1.76 mm, and 21.9$\pm$2.54 mm, respectively, and average size of small, medium and large females 20.5$\pm$3.90 mm, 27.1$\pm$3.88 mm, and 35.2$\pm$3.29 mm respectively. Fish were housed together in 20-l plastic boxes with

members of the same sex and size class for about one week prior to observations. Small males reared in the laboratory were checked daily for maturation (formation of the gonopodium; Snelson 1989) and held in isolation until behavioral observations. Time to maturity in male *G. affinis* varies from about 21-90 days, for both large and small males (Deaton unpublished data). I found no significant correlations between effects of either age at sexual maturity (presence of gonopodium) on mating attempts (R^2=0.002, N=24, p=0.83) or the number of days after sexual maturity on mating attempts (R^2=0.00, N=24, p=0.935) for small males reared in the lab.

The experiment was conducted in July 2005, peak reproductive season for *G. affinis* in my population. I used a free-swimming (Houde 1997), "no choice" (or "forced choice"; Shackleton 2005) experimental design to test the effects of male and female body size on male mating attempts (number of times males thrust the intromittant organ toward the female genital pore). One male and one female were placed together for each behavioral trial. Although "choice" experiments may be a more realistic setting for mate choice studies, data are confounded by the presence of more than one female per replicate, causing non-independence of data (Houde 1997). A no-choice design allows for the control of such confounding variables, and allows for full contact between the focal male and female (Shackleton 2005).

Experimental Design and Statistical Analyses

I used a two-way ANOVA design to test the effects of female and male body size on male mating behavior. Prior to each behavioral trial, I randomly drew from nine possible male-female size combinations (x 10 replicates for total of 90 observations; Table 1). Each male-female pair was placed in a 20-l aquarium and allowed to acclimate

together for at least 10 min prior to observations (Houde 1997). The bottom of each tank was lined with a standardized amount of gravel (about 2 cm). I measured male mating attempts (# gonopodial thrusts) during a 5-min focal observation period for each replicate pair. Because trials were conducted in early morning and evening (peak times for mosquitofish sexual behavior; Clark Hubbs pers comm), I included a time of day block as an effect of male mating attempts in the two-way ANOVA model.

I also measured female behaviors toward males (to generate a dichotomous measure of female "interest level"), assigning "0" to females that showed little to no interest (chasing and/or approaching males $\leq$ 2 times, and mostly ignoring and moving away from males when approached) and "1" to females that approached or chased the male at least 3 or more times during the 5-min observation. I used one-way ANOVA to test for differences in female interest levels based on female size (SL) and male size (SL), and a two-sample t-test to determine whether male mating behavior differed toward interested and uninterested females. I also used logistic regression to test for correlations between female size and interest levels.

During each behavioral trial, I recorded the size of the female gravid spot on a scale from 0-4 (0=no spot to 4=largest spot). The gravid (or pregnancy) spot is a dark (black) pigment spot on the abdominal region that forms as females develop ripe eggs (Farr and Travis 1986) and is thought to be a fertility indicator to males (Snelson 1989). Following each behavioral trial, both fish (male and female) then were stunned in ice water and immediately preserved in 5% formalin. I measured body size (SL) and girth to the nearest 0.5 mm, removed the ovary, and counted and staged all eggs and embryos (following Meffe's six-stage scale; 1985). Viscera were placed back into the carcass and

specimens were dried at 40° C to constant dry weight (10 days). To assess body condition, carcasses were weighed to the nearest 0.001g, rinsed six times overnight in petroleum ether to extract soluble fat, dried again overnight at 40° C, and reweighed. Condition was quantified following Marsh-Matthews et al. (2005) from residuals of linear regression of mass somatic fat (calculated as pre-extraction mass minus post-extraction mass) and initial mass (or pre-extraction mass).

Using those individuals for which all post-experimental measurements were available (N=80), I measured 10 male and female characteristics (Table 2). I used two statistical approaches to address the effects of body size and other correlated variables on male mating behavior: stepwise multiple regression using residuals and path analysis. Because several male and female characteristics were correlated with body size (Pearson correlation, $r \geq 0.65$; $p < 0.01$), I used residuals of regressions of correlated traits as independent variables. The residual approach is used in structural linear modeling to remove correlations between independent variables (Brown & Prescott 1999), which otherwise would violate the assumptions of multiple regression (Sokal & Rohlf 1995). Pilastro et al. (1997) found that size of the male relative to female was the best predictor of male mating success in the closely related *G. holbrooki*; therefore, I also included the absolute difference in male and female size in the model. Because this difference is a function of female SL and male SL, I conducted two multiple regressions, the first including female SL and male SL and the second including the absolute difference between female and male SL. Number of mating attempts (gonopodial thrusts) was the response variable. Analyses were conducted in SPSS version 13.0 for Windows, and SAS (SAS Institute 2000).

For path analysis (Wright 1921), I created a set of 32 *a priori* models that reflect hypothetical relationships between female and male characteristics and male mating behavior (Johnson 2002; Table 3). All measured variables included in *a priori* models are depicted in a single global model (Fig. 1; Johnson 2002). I did not intend to include all variables and all interactions between variables in each path model. I generated models that I felt were realistic (and biologically significant) representative models that best predict male mating behavior. I focused on four major male characteristics, four major female characteristics, and the difference between male and female size (SL; see Table 2). Path models were generated using the software Amos 4.0 (Arbuckle and Wothke 1999) and were based on correlation matrices of measured variables on male mating behavior. I generated AIC values for each model in Amos 4.0 to assess model fit (Johnson 2002).

Results

Two-way analysis of variance showed no significant time of day block or male-female interaction effect on male mating attempts; therefore, I removed those factors from the model. When only male and female body size were examined, the overall model was significant (two-way ANOVA, $F_{4,89}=3.28$, $p=0.0148$, Table 4), with female size as the only significant factor ($p<0.01$; Fig. 2). There was no significant effect of male size on male mating behavior.

Female interest levels differed across male size treatments (one-way ANOVA, $F_{2,89}=8.256$, $p=0.001$, Fig. 3a). Tukey post-hoc tests showed that female interest levels were greater toward large than small males ($p<0.05$), but not toward medium sized males. Female interest differed significantly across all female size treatments (ANOVA, F

$_{2,89}$=17.214, p<0.001, Fig. 3b) but did not affect male mating attempts. Also, using 87 females for which continuous size measurements were made, female SL accounted for a significant amount of the variation in female interest levels, where smaller females showed greater interest than larger females (logistic regression, X^2=19.8, df=1, N=87, p<0.001, R^2=0.29).

For the 80 individuals for which all other measurements were available, female SL (p<0.001; Fig. 4a), testes mass (p<0.001; Fig. 4b), and size of gravid spot (p=0.03; Fig. 4c) significantly predicted male mating attempts (stepwise multiple regression, R^2=0.272, N=80, p<0.001). In a second stepwise multiple regression, the difference in male and female SL (p<0.001), testes mass (p<0.01) and size of gravid spot (p=0.02) were significant predictors (R^2=0.234, N=80, p<0.001). All other variables were removed from both models due to non-significance.

The most predictive path model included the difference between male and female size (p<0.001), male body condition (p<0.05), and testes mass (p<0.001) as significant predictors of male mating attempts (R^2=0.27, N=80, p<0.001; Fig. 5). The best model was chosen based on a combination of AIC and R^2 value (Table 3).

Discussion

Male preference for larger females is reported for many species (Werner & Lotem 2003), including fishes (Sargent et al. 1986), and expected when there is a strong female size-fecundity relationship (Pelabon et al. 2003). My study confirms that female size is an important predictor of male mating behavior in the western mosquitofish, *G. affinis*. Similar results have been reported for other livebearers, including the one-sided livebearer, *J. multidnetata* (Bisazza et al. 2000), guppy, *P. reticulata* (Herdman et al.

2004), and sailfin molly, *P. latipinna* (Ptacek & Travis 1997). However, Herdman et al. (2004) showed that female size is positively correlated with multiple paternity in the guppy, *P. reticulata* and suggested that a male's preference for larger females might increase susceptibility to sperm competition.

Male size, on the other hand, was not an important predictor of male mating attempts, which is contrary to findings reported for other livebearers, including *J. multidnetata* (Bisazza et al. 2000), where male size *and* female size were important predictors of male mating behavior (Bisazza et al. 2000) and for *Brachyrhaphis rhabdophora*, where small males preferred smaller females (Basolo 2004). Also, in the guppy and other livebearers, small males typically use sneaky tactics (forced copulations) and large males spend more time courting (Houde 1997), showing marked differences in mating behaviors of males based on body size. My results, however, did not show size-correlated mating behaviors by males, as reported by Hughes (1985). Bisazza and Marin (1995) indicated a negative correlation between body size and successful mating (measured as gonopodial thrusts) in the eastern mosquitofish, *G. holbrooki*, suggesting that small male body size is actually advantageous in that species. In my study, I found no difference in the number of mating attempts based on male size. Males of all size classes preferred larger females and small males did not avoid larger females. The path analysis, however, showed the size of the male relative to the female (measured as the difference between male and female SL) as the most important predictor of male mating behavior. Similar findings have also been reported for the *J. multidnetata* (Bisazza et al. 2000).

Female mosquitofish rarely initiate mating; however, female interest levels may provide some insight into female choice. My results show that female size strongly influences female interest levels. Interestingly, small females showed the highest interest levels, suggesting that young (and possibly) naïve females are more receptive to males. Roberts & Eutz (2005) showed that male wolf spiders preferred to mate with adult, un-mated (virgin) females, and suggested that males can assess potential receptivity of females. Bisazza et al. (2000) reported that females of the one-sided livebearer deprived of their sperm stores associated more with males, and also showed preferences for larger males. In this study, female interest levels increased with male size, showing that females were more receptive to larger males, but female interest levels did not influence male mating attempts. This may be because males prefer larger females, and larger females were not receptive to males. Hughes (1985), on the other hand, showed differences in small and large male mating behavior in *G. affinis* when exposed to receptive females. It has also been suggested that guppy males increase their sexual activity toward receptive females (Houde 1997). Most studies on the mosquitofishes have shown little evidence of overt female choice (but see Hughes 1985, 1986; McPeek 1992; Gould et al. 1999); however, less obvious (e.g. female receptivity) or other cryptic mechanisms (e.g. sperm choice) of female choice may be important (Bisazza et al. 2001). My results suggest that mating preferences of small females should be examined in more detail and female interest levels may be a good surrogate measure of female choice and/or receptivity in mosquitofish.

In addition, I used two statistical approaches to determine male and female characteristics that are important predictors of male mating. The best step-wise multiple

regression showed that female body size (rather than the difference in female and male size), size of the female gravid spot, and male testes mass significantly predicted the number of mating attempts by males. But the best path model (based on R^2 and AIC values) included only two predictive variables, the difference between male and female size and male testes mass. Both models were highly significant and accounted for 27% of the variation in male mating behavior.

Other than body size, size of the gravid spot and testes mass were important predictors of male mating. If the gravid spot is a fertility indicator for males (Peden 1973), my expectations would be two-fold. First, I would expect females with ripe eggs to have the largest gravid spots and, secondly, I would predict males to increase the number of mating attempts toward females with the largest spots. However, I found a negative correlation between the size of gravid spot and male mating. In addition, previous studies (Deaton unpublished data) have shown that the size of gravid spot is positively correlated with embryo stage (in contradiction to other reported results), and males avoid mating with females close to parturition (Deaton unpublished data). Female mosquitofish can store sperm for up to several months (Constantz 1989), and sperm quality has been shown to decrease over time in mosquitofish (Hildebrand 1917 cited in Hughes 1985) and other species (e.g. birds; Wagner et al. 2004). Thus, the negative correlation between male mating and the size of the gravid spot may be a result of males avoiding those females with late stage broods (no ripe eggs for immediate fertilization), which may be an evolutionary strategy used by males to avoid reduction in fitness via reduced fertilization success. To my knowledge, this has not been tested in livebearing fishes.

Testes mass also was a significant predictor of male mating attempts. Testes mass is usually highly correlated with male size, but I corrected for this correlation in my analyses. In mosquitofish, larger males are more aggressive (Hughes 1985), but larger males in my study populations do not have larger testes per unit SL than smaller males (Deaton unpublished data). Testes mass is likely correlated with male hormones driving sexual behavior (e.g. testosterone, 11-ketotestosterone; Borg 1994), which would explain why males with larger testes relative to body size attempt to mate at higher rates.

In this study, my models accounted for a significant amount of variation in male mating behavior, but considerable variation was left unexplained. Nonsignificant variables included female girth, male body size (previously discussed), female and male condition, and gonopodial length. It was surprising that female girth (as a surrogate for fecundity) did not influence male mating behavior. In the one-sided livebearer, Bisazza et al. (2000) showed that males did not discriminate between gravid and non-gravid females. But, Park and Propper (2001) showed that male mosquitofish change their sexual behavior based on the reproductive state of females. Also, if males prefer to mate with larger females because they are more fecund, it might be expected that they would cue in on girth as a measure of size. Because body condition, as measured in this study (as soluble fat stores), varied little among individuals (all individuals were fed *ad lib*), it is not surprising that body condition did not influence male mating behavior. Finally, Langerhans et al. (2005) showed considerable size variation in gonopodium length among species and populations of mosquitofish, and that females associated more with males with longer gonopodia, both of which suggest that gonopodial length may be under sexual selection. In this study, there was little variation in gonopodial length (after

correcting for male size; Deaton own data), suggesting that gonopodial length may not be important for male mating success in all populations.

Importantly, this study illustrates that factors other than female size are important predictors of male mating behavior in the western moquitofish. My results share similarities and differences with those reported for the related one-sided livebearer (Bisazza et al. 2000) but corroborate that female body size (or male size relative to female size) is perhaps the most important predictor of male mating behavior. Livebearing fishes are an ideal system to make such comparisons because several mechanisms of sexual selection (e.g. female choice, male-male competition, sexual coercion) may be at play at any given time. This leaves a challenge to researchers studying factors influencing male mating behaviors in species with coercive mating systems. Such systems are unquestionably complex and deserve considerably more attention to better understand mechanisms of sexual selection and factors influencing male reproductive success.

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Table 1. Randomly drawn male-female mating pairs based on size (small=1, medium=2, large=3) for two-way ANOVA. Each of nine pairs (treatments numbers 1-9) represents one mating observation per behavioral trial. Each treatment was replicated 10 times, for a total of 90 behavioral observations.

Mating combination (Size Pair)		Treatment
Male Size Class	Female Size Class	
Small	Small	1
Small	Medium	2
Small	Large	3
Medium	Small	4
Medium	Medium	5
Medium	Large	6
Large	Small	7
Large	Medium	8
Large	Large	9

Table 2. Selective agents included in the path analysis model defined by their hypothetical effects on male mating behavior. Path numbers and abbreviations (denoted in parentheses) correspond to those diagrammed in Fig. 1.

Hypothesis	Path
Direct Effects	
Female Characterisitcs	
Body size (F-SL mm)*	1
Female condition (F-condition)*	2
Female girth (F-girth mm)*	3
Fecundity (embryo number)	
Embryo Stage	
Size of gravid spot (F-GS)*	4
Male Characteristics	
Body Size (M-SL)*	5
Body Condition (M-condition)*	6
Testes Mass (M-TM)*	7
Gonopodial Length (M-GL)*	8
Male body size relative to female body size* (M-F SL Difference mm)	9
Unknown Effect	
Variation in male mating that cannot be explained by the variables included in the model.	10

*Asterisks denote independent variables included in stepwise multiple regression models.

Table 3. An a priori set of 34 candidate models that denote biologically significant hypotheses to explain male mating behavior in *G. affinis* (measured as # gonopodial thrusts). Selective agents are as follows: Female = female characteristics; Male = male characteristics; and Female/Male = both male and female characteristics. Models are defined as the path numbers shown in Fig. 2. Asterisk (*) indicates the representative best path based on a combination of AIC and R^2 values. Sample sizes (*n)* represent the number of replicates tested to generate AIC values and R^2 values in each path model.

Selective agents	Model (defined by paths)	AIC *n*=81	R^2 *n*=81
Female	1,10	6.0	0.11
Female	2,10	6.0	0.02
Female	3,10	6.0	0.04
Female	4,10	6.0	0.00
Female	1,2,10	12.0	0.13
Female	1,3,10	12.0	0.15
Female	1,4,10	12.0	0.13
Female	2,3,10	12.0	0.07
Female	2,4,10	12.0	0.02
Female	3,4,10	12.0	0.06
Female	1,2,3,10	20.0	0.16
Female	1,2,4,10	20.0	0.14
Female	1,3,4,10	20.0	0.16
Female	1,2,3,4,10	30.0	0.16
Male	5,10	6.0	0.02
Male	6,10	6.0	0.06
Male	7,10	6.0	0.10
Male	8,10	12.0	0.00
Male	5,6,10	12.0	0.09
Male	5,7,10	12.0	0.12
Male	5,8,10	12.0	0.03
Male	6,7,l0	12.0	0.16
Male	6,8,10	12.0	0.06
Male	7,8,10	12.0	0.12
Male	5,6,7,10	20.0	0.17
Male	6,7,8,10	20.0	0.18
Male	5,7,8,10	20.0	0.12
Male	5,6,7,8,10	30.0	0.18
Female/Male	1,6,7,10	17.45	0.23
Female/Male	9,6,7,10*	20.0	0.30
Female/Male	1,6,7,8,10	25.59	0.24
Female/Male	9,6,7,8,10	30.0	0.27
Female/Male	9,2,3,4,6,7,8,10	62.78	0.33
Female/Male	1,2,3,4,6,7,8,10	65.46	0.28

Table 4. Two-way analysis of variance showing F-statistic and associated level of significance for female size (SL) and male size (SL) effects on male mating attempts (number of gonopodial thrusts).

Source of variation	*df*	F-value	Pr>F
Overall Model	4,89	3.28	0.0148
Male Size Effect (SL)	2,89	0.045	0.6548
Female Size Effect (SL)	2,89	6.14	0.0032

Figure 1. A global model path diagram depicting putative selective agents on male mating attempts in the western mosquitofish, *G. affinis* (represented by shaded square in the center of diagram). Selective agents in boxes represent measured traits (both male and female) included as independent variables in both path analysis and multiple regressions. Variation in the model unexplained by the 9 selective agents is represented by the unknown effects (shown in unshaded circle). Numbered arrows represent regressions of the selective agents on male mating behavior. Single-headed arrows represent direct effects of selective agents on male mating behavior and double-headed arrows depict correlations among independent variables included in hypothesized *a priori* path models (shown in Table 3). Abbreviations for selective agents are explained in Table 2.

Figure 2. Average number of male mating attempts (measured as # gonopodial thrusts; GT) by males toward females from each size class (small, medium, large). Letters above bars represent significant differences between each size class (Tukey post-hoc comparisons, $p<0.01$). Error bars represent Mean+1 SE (N=90).

Figure 3. Average female interest level (measured dichotomously as 0 or 1) of females toward males in different size classes (a) and of females in different size classes (b). Letters above bars represent significant differences between each male (a) and female (b) size class (Tukey post-hoc comparisons, $p<0.01$). Error bars represent Mean+1 SE (N=90).

Figure 4. Male mating attempts (measured as # gonopodial thrusts; GT) as a function of female size (a), size of female gravid spot (b), and testes mass (c) for 80 males included in a stepwise multiple regression model (MMB=FSL(0.218) - PS(1.532) + TM(5.434) – 1.493; $R^2 = 0.272$, df=3,79, F=9.588, $p<0.001$).

Figure 5. The most predictive path model (based on a combination of R^2 and AIC values) from all alternative *a priori* hypotheses (path models), showing significant predictors of male mating behavior (number of mating attempts). Single-headed arrows represent direct effects on male mating, and numbers below lines represent path coefficients. Double-headed arrows represent correlations between independent variables, and numbers to the right of the lines represent correlation coefficients. Asterisks denote significance levels at $p<0.05$*, $p<0.01$**, and $p<0.001$***. R^2 value above dependent variable (shown in shaded square box) represents total amount of variation explained in model. Unexplained variation is shown in the unshaded circle (U).

Figure 1

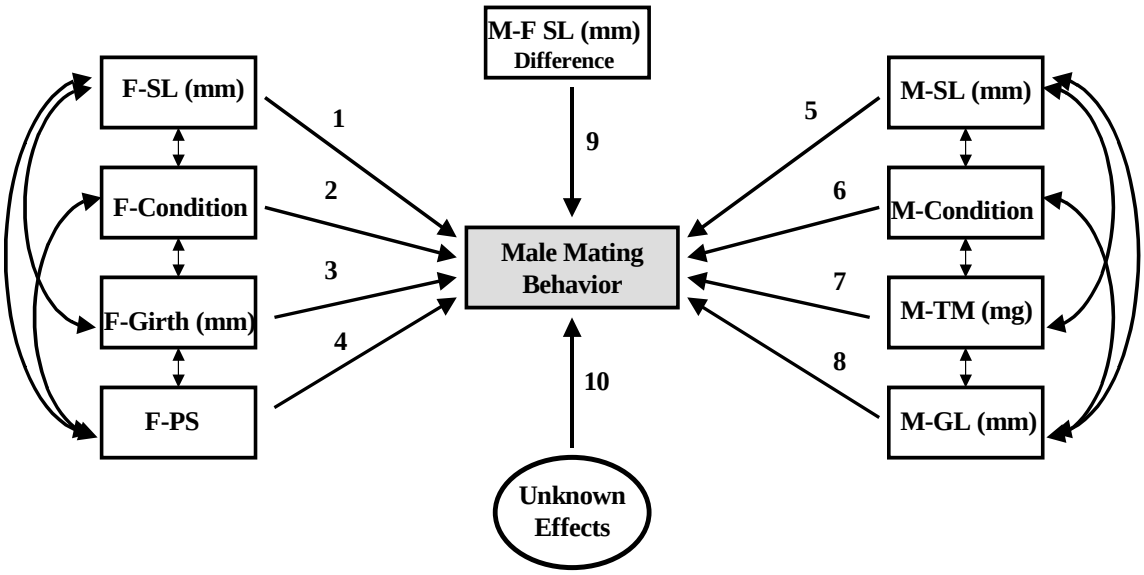

M-F SL (mm)
Difference
F-SL (mm)
F-Condition
F-Girth (mm)
F-PS
M-SL (mm)
M-Condition
M-TM (mg)
M-GL (mm)
Male Mating
Behavior
Unknown
Effects
1
2
3
4
5
6
7
8
9
10

Figure 2


Mean # Gon. Thrusts (GT)
10
8
6
4
2
0
A
A,B
B
Small
Medium
Large
Female Size Class

Figure 3

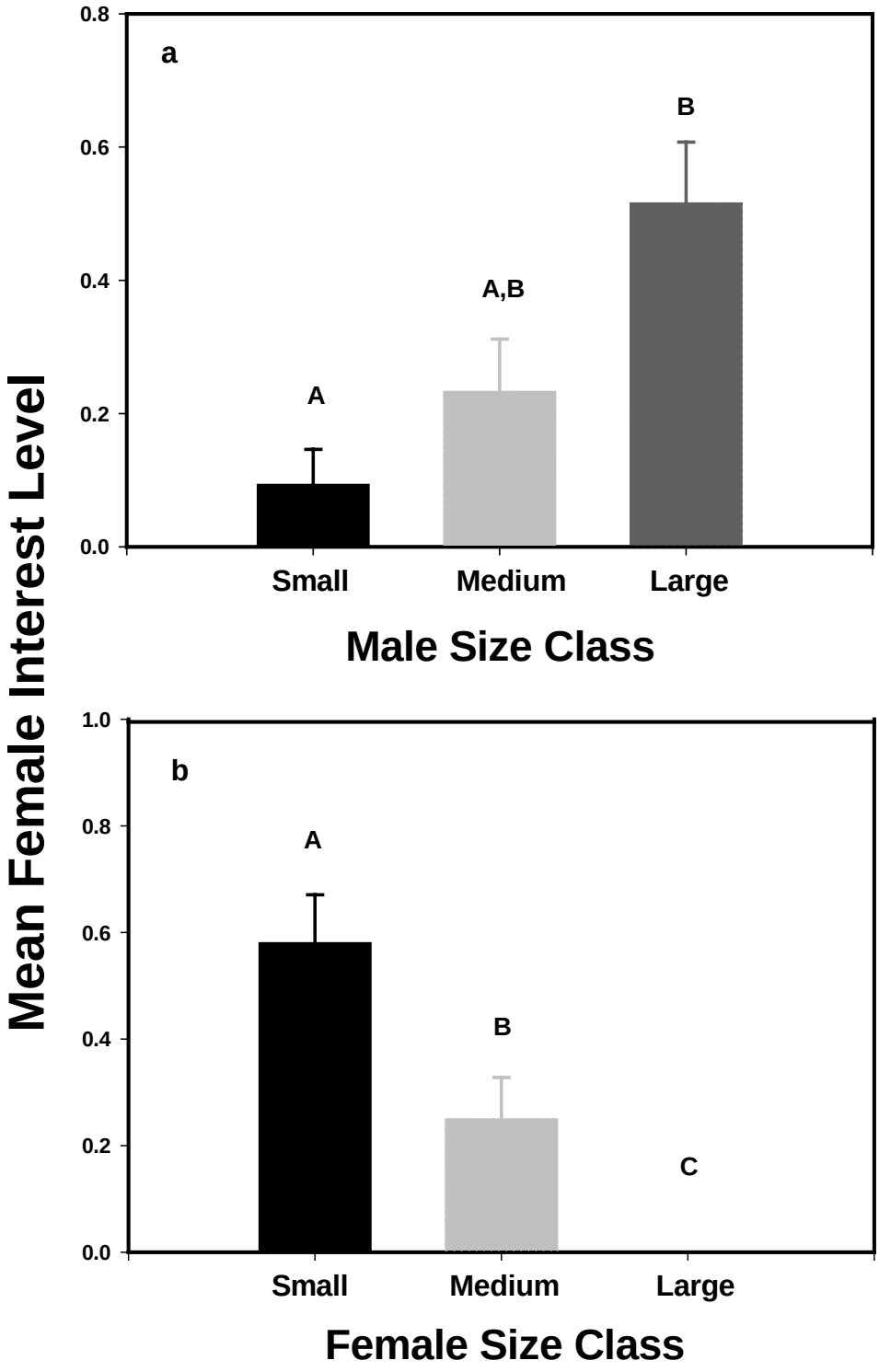

a
0.8
0.6
0.4
0.2
0.0
A
A,B
B
Small
Medium
Large
Male Size Class
Mean Female Interest Level
b
1.0
0.8
0.6
0.4
0.2
0.0
A
B
C
Small
Medium
Large
Female Size Class

Figure 4

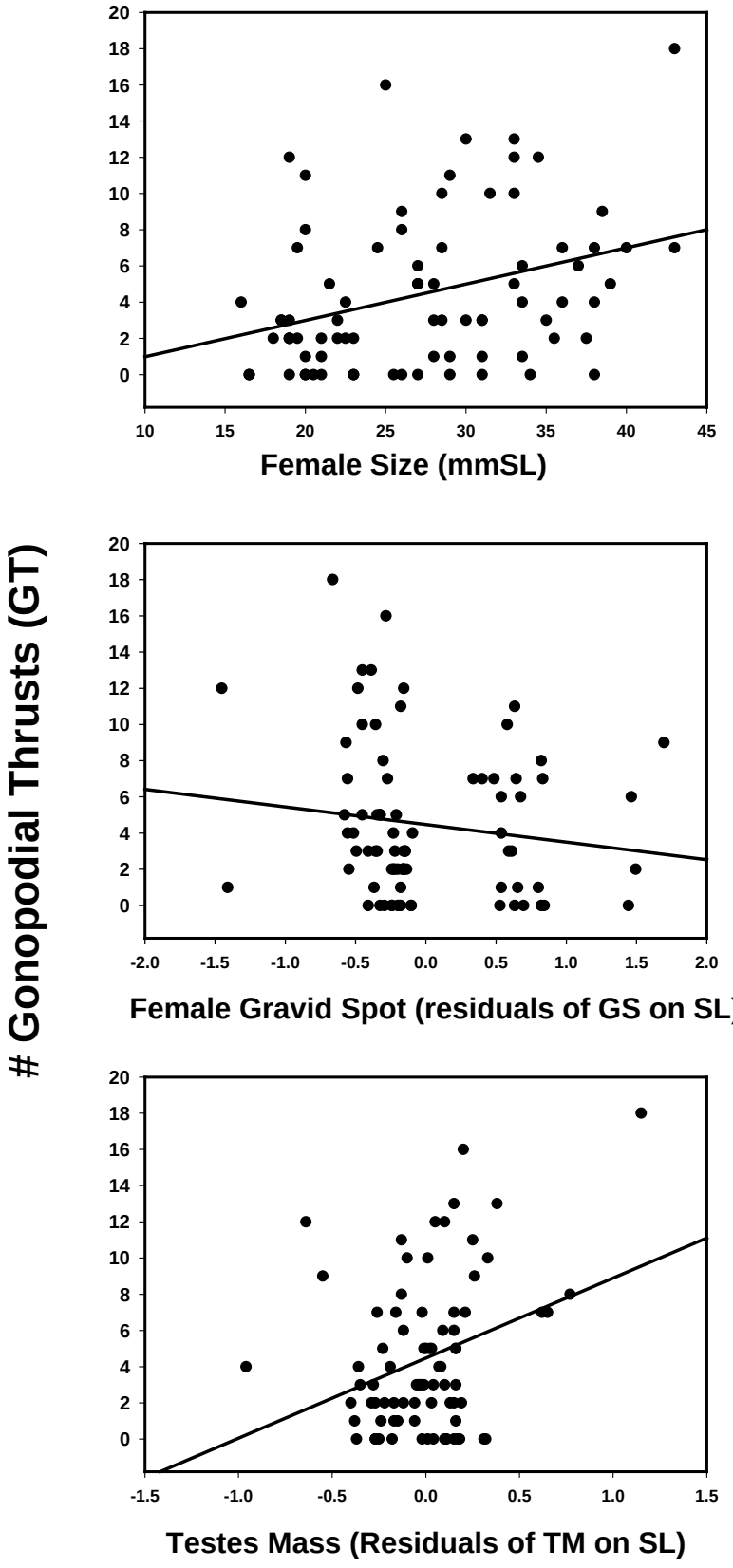
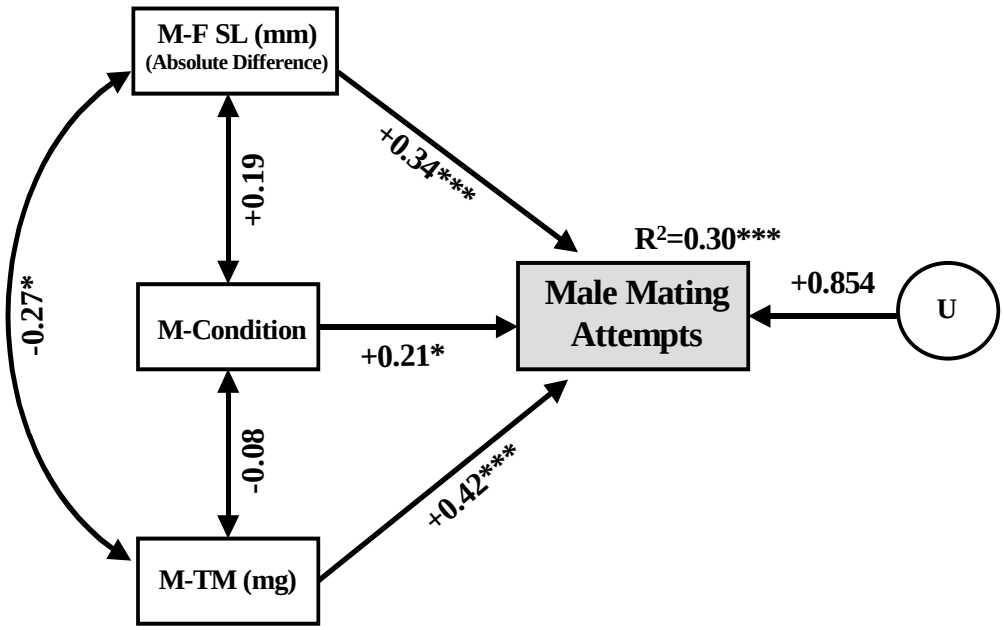

20
18
16
14
12
10
8
6
4
2
0
10
15
20
25
30
35
40
45
Female Size (mmSL)
Gonopodial Thrusts (GT)
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
Female Gravid Spot (residuals of GS on SL)
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
Testes Mass (Residuals of TM on SL)

Figure 5


M-F SL (mm)
(Absolute Difference)
+0.19
+0.34***
R2=0.30***
-0.27*
M-Condition
Male Mating Attempts
+0.854
U
+0.21*
-0.08
+0.42***
M-TM (mg)

CHAPTER 2

Do parasites mediate sexual selection in fish with a coercive mating system?

Raelynn Deaton

Abstract

Parasites can strongly influence mating decisions in many organisms, particularly in species where females choose mates based on elaborate secondary sexual characteristics. In this study, I tested parasite-mediated sexual selection in the western mosquitofish, *Gambusia affinis*, an organism in which sexual coercion, rather than mate choice, is the more important mechanism of sexual selection. In two separate experiments, I tested the effects of a parasitic nematode on female mate choice, male mating behaviors, male-male competition, and male mate choice. I predicted that both females and males would mate preferentially with nonparasitized individuals of the opposite sex. I also predicted parasitized males would be less competitive for mates than uninfected males. I found that females showed no significant mating preferences toward uninfected males. Further, parasitized males were not in reduced body condition and the presence of the parasite did not alter male aggression or mating behavior. Males, on the other hand, did exhibit preference for uninfected females, and the level of male mating behavior varied inversely with the relative mass of parasites in infected females. Uninfected females were in marginally better condition than infected females, but female condition was not a function of relative parasite mass. This study shows that parasites may be an important driving force in male mate choice, even in species where mate choice is not the primary mechanism of sexual selection.

Introduction

Hamilton and Zuk's (1982) influential paper on parasite-mediated sexual selection sparked considerable attention in behavioral and evolutionary ecology. Since then, a wealth of research has been dedicated to understanding the effects of parasites on mate choice (see Moller, 1990; Barber, 2002; Moore, 2002). However, most studies have focused on female choice for males with obvious or exaggerated secondary sexual characteristics, such as bright color patterns of many birds (Hamilton & Zuk, 1982; Zuk *et al.*, 1990; Johnson, 1991; Sundberg, 1995; Weihn et al., 1997) and fishes (Kennedy et al., 1987; Milinski & Bakker, 1990; Bronseth & Folstad, 1997; Lopez, 1999), presumably because those traits signal parasite resistance which may be passed onto offspring (Hamilton & Zuk, 1982; Moller, 1990; Bronseth & Folstad, 1997). Although the effect of parasites on mating behaviors is well studied, results are often contradictory, and vary among parasite species, host species, and parasite virulence (see Moller, 1990; Moore, 2002).

Parasite effects also may differ considerably across mating systems. For example, parasites may be important in mediating sexual selection in organisms where female choice for male characteristics is strong (i.e. guppies, sticklebacks), but only if parasites significantly reduce male fitness or change phenotypic cues important for mating. In such organisms, a negative correlation between male mating success and parasite load is expected (Forbes, 1991). Parasites can influence male reproductive success by affecting female choice (by reducing male condition, degree of ornamentation, or courtship frequency) or by reducing competitive abilities of parasitized males. For example, Kennedy et al. (1987) showed a negative correlation between number of parasites and the

number of male sexual displays in the guppy, suggesting that parasite infection indirectly decreased male fitness by reducing frequency of courtship behaviors. Barber (2002) showed a negative correlation between the size of the dorsal fin and the number of ectoparasites in the sand goby, and suggested that parasite infections may affect important phenotypes used by females in mate choice. Parasites also may reduce male competitive ability by reducing condition, thus, causing males to put more energy into foraging than into mating (Forbes, 1991). In some species, such as the two-spotted goby, parasites have been shown to have no effect on male condition, but parasitism is correlated with a significant decrease in male courtship intensity (Pelabon et al., 2005).

Despite the wealth of knowledge on parasite effects on sexual selection, research is lacking in two areas: (1) the effects of parasites on male mate choice; and (2) the effects of parasites in coercive mating systems, where mechanisms other than mate choice (i.e. forced copulations) also are important components of sexual selection. Coercive mating systems, where males of all sizes use forced copulations as their primary means of obtaining mating success, are known in a wide variety of taxa, including monarch butterflies (Solensky, 2004), water striders (Arnqvist & Rowe, 1995), garter snakes (Shine & Mason, 2005), bush crickets (Vahed, 2002), and some species of the livebearing fish family Poeciliidae (e.g. mosquitofishes; Bisazza et al., 2001). Mate choice also may be present in these systems, but male coercion is considered to be the primary mechanism of sexual selection.

Mosquitofish provide an excellent opportunity for studying parasite-mediated sexual selection in a coercive mating system, because many potential mechanisms of sexual selection may be operating, including male choice and some level of female mate

choice, variation in male mating behavior, and male-male competition (Hughes, 1985). Mosquitofish (*Gambusia spp*.) are small, livebearing fish in which males of all sizes force females to copulate (Bisazza et al., 2001). It has been suggested that males mate indiscriminately (Bisazza & Marin, 1991,1995), and female choice is negligible (Hughes, 1985, McPeek, 1992), but recent studies have shown that males prefer larger females (Deaton, in review). Although females usually do not show overt mating preferences (but see Gould et al., 1999; Langerhans et al., 2005), they may control mating via other, less obvious mechanisms such as resistance (Deaton, pers. obs.) and receptivity (Bisazza et al., 2001). According to parental investment theory, female mosquitofish should be the choosier sex because they have higher relative investment in offspring (Trivers, 1972). However, this does not appear to be true in mosquitofish, where most evidence points to males being the more discriminatory sex (Hughes, 1985; Bisazza et al., 1989; Deaton, in review). Thus, if parasites affect mate choice (male or female) at any level, they could actually have a greater effect on intersexual selection than might be expected for a coercive mating system where mate choice generally is thought to be weak or even absent.

Unlike most fishes, mosquitofish exhibit internal fertilization, where males use an intromittant organ (gonopodium) to transfer sperm to the female. Thus, males and females make direct contact when mating, potentially intensifying the effects of parasites on mating behaviors, assuming parasites alter morphological, behavioral, and/or physiological cues used for mating. Further, because male mosquitofish do not exhibit elaborate secondary sexual characteristics to attract females, such as the bright color patterns of male guppies (Houde, 1997) or exaggerated tails of male plattyfish (Basolo,

2002), female choice, to the extent that it exists, may be based on other male characteristics (i.e. size, body condition, parasite load) to determine male quality. Finally, some populations of the western mosquitofish (*G. affinis*) across Oklahoma and Texas are heavily infected with the gastro-intestinal parasitic nematode *Eustrongylides ignotus* (Coyner, 1998), which has negative effects on reproduction in the western mosquitofish (Brooks, 2005; Deaton, unpublished data). Decreased female fecundity due to parasites may have pronounced consequences for female reproductive success if males avoid mating with infected females. For example, males may resort to mating with smaller, less fecund females, possibly resulting in marked differences in mating dynamics and mechanisms of sexual selection between parasitized and unparasitized populations of mosquitofish.

The lifecycle of *E. ignotus* is fairly complex, because it relies on several hosts throughout its development. Its primary host is a sediment-dwelling oligochaete, which is consumed by an intermediate host (e.g. mosquitofish or other vertebrate species). The nematode then matures and reproduces in a terminal host (a piscivorous bird; Coyner, 1998). This parasite is transferred only horizontally through consumption of infected individuals. Because *E. ignotus* can reach up to 50% of the body mass in mosquitofish (Deaton, Brooks, Marsh-Matthews, unpublished data), negative consequences for host reproduction, susceptibility to predation, and/or mating behaviors are expected. Coyner et al. (2001) showed that *G. affinis* individuals infected with *E. ignotus* are more susceptible to predation by birds and Brooks (2005) found that *E. ignotus* decreased female reproduction by increasing inter-brood interval. Further, I found that female *G. affinis* infected with *E. ignotus* had fewer embryos than uninfected females (Deaton, in

prep). Clearly, this parasite alters morphology and fecundity of the western mosquitofish, leading to expectations that it may also have substantial effects on mating behavior.

In two separate behavior experiments, I examined the effects of a parasitic nematode on sexual selection in the western mosquitofish, *G. affinis.* I tested parasite effects on both female and male mate choice, male mating behavior, and male-male competition. I predicted that both females and males would mate preferentially with nonparasitized individuals of the opposite sex and that parasitized males would be less competitive for mates than uninfected males. To examine possible parasitic effects underlying mate choice, I assayed body condition (fat reserves) of parasitized and nonparasitzed individuals and evaluated reproductive condition of the females that were subjects in mate choice experiments.

Methods

During August and September, 2003, I made weekly collections of mosquitofish in a small pond in Norman, Oklahoma. The mosquitofish population in this pond is parasitized with *E. ignotus*, although the parasite rate fluctuates annually (Deaton et al., unpublished data). In 2003, infection rate was about 35%. Fish were returned to a greenhouse laboratory and acclimated in two 340-l community tanks for several weeks and fed commercial flake food once daily.

I performed both female mate choice and male mate choice experiments using an open water experimental design (Houde, 1997) because it allows fish to swim freely throughout the tank and closely inspect the other individuals. Behavior observations

were conducted in October and November, 2003, late in the breeding season, to allow for enough parasite growth to visually detect infected individuals.

All behavioral trials were taped on a digital video camera during observations. Using video footage, two observers recorded association time of the male toward each female during a ten-minute period. Female treatment (parasitized or nonparasitized) was randomly assigned to each observer prior to behavioral trials to control for potential observer biases. Association time was measured as males being in close vicinity to the females (within 2 cm). Prior to the experiment, a pilot study was conducted on a few individuals to assure consistency in data collection between the two observers.

In addition to behavioral observations, I compared characteristics of the parasitized and nonparasitzed individuals subject to choice in each experiment. In the female choice experiment, I assayed male body condition, male mating intensity, and male-male competition (measured as frequency of aggressive acts). For the male mate choice experiment, I examined female body condition and reproductive state.

Individuals were euthanized in MS-222 immediately following behavioral observations, and preserved in 10% formalin until dissection. Preserved specimens were measured (to the nearest 0.5 mm standard length; SL), dissected, weighed, and subjected to fat extractions (for condition analysis).

To assay body condition, carcasses and livers were weighed to the nearest 0.001 g, rinsed six times overnight (or longer) in petroleum ether to extract soluble nonstructural fats (Heulett et al., 1995; Trexler, 1997), dried overnight at 40ºC, and reweighed. Body condition was quantified as the standardized residual from least squares linear regression of mass of somatic fat (calculated as pre-extraction mass minus

post-extraction mass) on pre-extraction mass (Marsh-Matthews et al., 2005). Because female dry mass is a function of female size (SL), and female size and condition are often correlated (Deaton, in review), I used ANCOVA to test the effects of parasites on female mass and condition, using female SL as a covariate. For this analysis, I used 51 females (40 of which were test subjects in male mate choice experiment) for which dry mass and condition data were available.

The ovary was removed from each female in the male mate choice experiment (see below), embryos counted and separated (based on Meffe's six-stage scale, 1985), dried at 40ºC for 10 days, and weighed (to the nearest 0.001g). Parasites were also removed from females, dried, and weighed to calculate a parasite index (percent eviscerated parasite body mass to eviscerated female body mass) to test for correlations between parasite index, body condition and male mating behavior. All statistical analyses were conducted in SPSS 13.0 for Windows.

Experiment I: Female Mate Choice, Male Mating Behavior and Male-Male Competition

Female Mate Choice

To test the hypothesis that parasites affect female mate choice in the western mosquitofish, I chose one parasitized and one nonparasitized male from community tanks and placed them in a 30-l aquarium with one randomly selected female, for a total of 13 replicates (or 13 females and 26 males). Each replicate included one randomly selected female with a parasitized and a nonparasitized male. Males were difficult to collect from the field, hence the small sample size for this experiment. Parasitized individuals are readily identifiable once the parasite reaches a certain size (approximately 10% of host body mass) because of the asymmetrically shaped abdomen of infected individuals.

After a 10-minute acclimation period, I made 8-minute focal observations for each male (Houde, 1997). Following the experiment, males were returned to their community tanks for future use.

To test the prediction that females show mating preferences toward nonparasitized males, I measured female mating behaviors and association time with each male. Females do not typically initiate matings, but they do orient toward, approach, circle, and chase males, behaviors that may indicate mating interest toward males (Deaton, in review). Female also ignore males (turn or swim away when approached) and/or swim to the bottom of the tank, possibly to avoid male harassment. I recorded four female behaviors: ignore (measured as the female turning away from a male when approached), orient (measured as a female turning toward a male, but not followed by a chase), circle, and chase. Because I only observed circling behavior by one female, I did not include circling in analyses. Orient and chase were significantly correlated (Pearson's $R=0.314$, $df=12$, $p<0.05$); therefore, I summed the two behaviors to create a response variable for female preference (see methods Husak and Fox 2003).

Male Mating Behavior and Male-Male Competition

To test the prediction that nonparasitized males exhibit higher frequencies of mating behaviors, I recorded each male's mating behaviors following the same experimental protocol as described above. Male mating behaviors included orient (measured as male turning toward female, but not ending in a chase), chase, circle, swim under (or positioning), lateral display (lowering the gonopodium), nipping (usually at the genital opening), and copulation attempt (measured as number of times males thrusts the gonopodium toward the female's gonopore; gonopodial thrusts; GT; Krotzer, 1990 and

Houde, 1997). Because male mating behaviors in mosquitofish are often correlated (Deaton unpublished data), I used a Pearson's correlation matrix to determine which behaviors were correlated with actual mating attempts in order to formulate a male mating response variable. Swim under was the only behavior highly correlated with actual mating attempts (Pearson's R=0.559, df=25, p=0.003). Chase and circle were both marginally correlated with number of mating attempts (0.33 and 0.33, respectively, df=25, p=0.09), and therefore, excluded from the analysis. Nipping was also excluded from analyses because the frequency of this behavior was very low (N=6). Therefore, I summed swim under and gonopodial thrusts to create a male mating response variable (see methods Husak and Fox, 2003).

To test the prediction that nonparasitized males are more aggressive, and thus, better competitors for mates, I measured all male aggressive interactions during each trial. I scored male-male aggression as a dichotomous variable (0 or 1) based on the number of aggressive behaviors made by males during the experiment. Commonly observed aggressive behaviors by males included back arch, gonopodial display, and chase (Krotzer, 1990), but chasing was the only aggressive behavior observed in this experiment. Males that chased the other male at least once during the observation period were assigned a "1" for aggression, and males that did not chase the other male were assigned a "0" for aggression.

Experiment II: Male Mate Choice

Male Mate Choice

To test the hypothesis that parasites affect male mate choice in the western mosquitofish, I chose one parasitized and one nonparasitized female from community

tanks and placed them in a 30-l aquarium with one randomly selected male. Other than presence/absence of parasites, I attempted to match females for size and phenotype [e.g. size of gravid spot, or dark pigmentation that appears in the abdominal region when female obtain ripe eggs (Snelson, 1989) and body pigmentation]. After a 10-minute acclimation period, I made 8-minute focal observations for each female (Houde 1997). During each behavioral trail, I recorded male mating behaviors toward each female (parasitized and nonparasitized; order of focal female was randomized), for total of 20 replicates. Male mating behaviors included orient, chase, circle, swim under, lateral display, nipping, and copulation attempt (or gonopodial thrust; see descriptions in Experiment I; Krotzer, 1990 and Houde, 1997). Swim under (or positioning) and copulation attempt were highly correlated (R=0.44, df=19, p=0.004); therefore, I used the sum of these two behaviors as my response variable (see methods Husak & Fox, 2003).

Results

Experiment I: Female Mate Choice, Male Mating Behavior, and Male-Male Aggression

Female Mate Choice

Females showed no mating differences between nonparasitized and parasitized males (paired t-test, t=1.414, df=12, p=0.18). Females did not ignore parasitized males at higher rates than nonparasitized males, (paired t-test, p=-0.69, df=12, p=0.5) nor did they associate more with nonparasitized males (paired t-test, t=1.008, df=12, p=0.33)

Male Mating and Male-Male Competition

Parasitized and nonparasitized males did not differ in frequency of mating behaviors (paired t-test, t=0.44, df=12, p=0.33), or competitive ability (measured as aggression; paired t-test, t=1.477, df=12, p=0.18). Aggressive behaviors were observed

in only 8 of 23 males. Of the 8 males showing aggressive behaviors, 6 were nonparasitized and two were parasitized.

Condition

Male body condition did not differ between treatments (two-sample t-test, t=-0.09, df=11, p=0.92); however, I was only able to measure male condition for 12 of the 26 males for this experiment. Statistical power for this measure is low; therefore, no further analyses were conducted on male condition.

Experiment II: Male Mate Choice

Male Mate Choice

Males mated more often with nonparasitized than parasitized females (paired t-test, t=-2.57, df=19, p=0.018; Figure 1a), but did not preferentially associate with nonparasitized females (paired t-test, t= -1.36, df=19, p=0.18; Figure 1b).

Female Characteristics

Nematode dry mass of parasitized females ranged from 1.064g – 19.2g, and averaged 9.93 $\pm$ 5.34g. Parasite index ranged from 3.51% – 31.72%, and averaged 16.25 $\pm$ 5.34%. Using 16 females for which parasite index was available (due to mortality of 4 individuals), a regression analysis showed that female parasite index was not a good predictor of female condition (R^2=0.018, df=15, p=0.59). However, female parasite index accounted for a marginally significant amount of variation in male mating (R^2=0.174, df=15, p=0.09; Figure 2). This relationship was negative, indicating that male mating behavior is inversely related to relative parasite mass of the female.

Nonparasitized females weighed more (two sample t-test, t=2.05, df=50, p=0.04), but were not larger (SL; two sample t-test, t=1.52, df=50, p=0.113) or in better condition

than nonoparasitized females (two sample t-test, t=1.59, df=50, p=0.117), although there was a trend towards nonparasitized females being both larger (SL) and in better condition.

ANCOVA was used to test for nematode effects on female mass, while correcting for female size. Results showed a significant female SL * nematode interaction (ANCOVA, $F_{1,50}$=5.496, p=0.023) and female SL was a significant covariate (ANCOVA, $F_{1,50}$=439.568, p<0.001). The overall model was significant (ANCOVA, $F_{2,50}$=238.558, p<0.001; Figure 4). There was no significant parasite effects on female condition (ANCOVA, $F_{2,50}$=1.131, p=0.346)

Only 6 females had developing embryos, all of which were nonparasitized females. No parasitized females had developing broods.

Discussion

This study shows that parasites mediate sexual selection in the western mosquitofish, mainly via male mate choice. Rate of parasitism in my study population for Summer 2003 was approximately 35%. For the females used in this experiment, parasite mass averaged about 16% of body mass of females (reaching up to about 32%). In the same habitat in 2002, I found *E. ignotus* in female mosquitofish reaching up to 50% of the body mass of the female, which led to expectations that this parasite may have significant consequences for female condition and/or reproduction. Although the relationship between parasites and female mass and body condition only approached significance in this study, parasitized females weighed less, were marginally smaller, and were in marginally poorer condition than nonparasitized females.

Several mechanisms could underlie the relationship between parasite infection and female size. *Eustrongylides ignotus* may preferentially infect smaller individuals, because after correcting for female size (SL), an analysis of covariance showed a significant interaction between presence of nematode and female size, indicating that parasites infect females of different sizes at different rates. Alternatively, female mass may have been reduced simply because parasitized females did not have developing broods, and thus, were not carrying yolky eggs and/or embryos. This is unlikely, however, given that there was a trend for smaller females to have a higher rate of parasitism. It is also possible that parasite infection retards the growth of female mosquitofish (which have indeterminate growth), rather than selectively infect smaller individuals. Further studies are needed to differentiate between these potential mechanisms for reduced growth and weight of parasitized females.

My results support, to some extent, the assumption that parasitized individuals suffer reduced body condition. In this study, I measured female condition as residuals of soluble fat stores (Marsh-Matthews et al, 2005). Prior to experiments, females were housed in the lab for several weeks and fed high quality commercial fish food *ad libidum*. It is possible that parasites did not dramatically reduce female condition due to abundance of resources. Brooks (2005) found that parasitized females were actually in better condition than nonparasitized females, when fish were fed daily for several weeks in the laboratory. Females may store fat for overwinter survival (Reznick and Braun, 1987) and for investment in future reproduction (Castellano et al. 2004). Brooks (2005) suggested that parasitized females may shift their reproductive strategies by investing more into future (by storing fat) rather than current reproduction (fecundity).

Parasitized females also were less likely to be reproductive. Brooks (2005) also showed that *E. ignotus* significantly reduced offspring production in *G. affinis*, by increasing inter-brood interval. In this experiment, only 6 females had developing broods, all of which were nonparasitized females. It is possible that parasitized females also are susceptible to shorter overall breeding periods due to reduction of resources available for current reproduction. The breeding season of *G. affinis* in my study population usually extends from mid-late March through late October or early November. This experiment was conducted late in the breeding season (October and November) because parasite growth continues throughout the season, and most parasitized individuals cannot be visually detected until late Summer (Brooks, 2005). Most females in this study may not have had developing broods because females were beginning to "shut down" reproduction before overwintering. However, males will mate later in the breeding season with females that are no longer reproductive (Deaton, pers. obs.). This may be important in parasitized populations of mosquitofish because males may use female girth as an indicator of parasite presence, especially later in the reproductive season when parasitism is more prevalent. Further studies are needed to test whether males use girth as an indicator of presence of parasites in females (and hence, female quality).

Results from female mate choice experiments suggest that females do not use parasites as a cue for male quality. However, because of the low sample size for male condition, I was unable to test the assumption that parasitized males were in poorer condition than nonparasitized males. Therefore, at this time, I do not know whether male condition was influenced by presence of parasites or whether this, in turn, influenced

female choice. In general, these results showed no behavioral or association differences toward infected and uninfected males, lending no support to the hypothesis that parasites influence female mate choice. These results contradict most studies in fish, where females prefer nonparasitized males over parasitized males (see Barber 2002). However, these studies measured female mating preferences in fishes where female choice for male secondary sex characteristics is an important mechanism in sexual selection. This is unlikely the case for mosquitofish, since most studies on mosquitofish mating behavior have reported little evidence for overt female choice (Bisazza & Marin, 1991, 1995; Bisazza et al., 2001).

In this study, parasitized and nonparasitized males did not differ in the frequency of mating behaviors, suggesting that parasites do not negatively affect a male's ability to mate. Pelabon et al. (2005) found that a microsporidian parasite did not decrease male body condition in the two-spotted goby, but did decrease courtship intensity. Parasites are known to reduce male condition in other fishes (Szalai & Dick, 1991; Heins, 2004), possibly having negative consequences for male mating success via reduction in competitive ability. However, in this study, parasitized males were no less aggressive than nonparasitized males, implying that nonparasitized males may not be better competitors for mates. Because sample sizes were low for this experiment, further work is necessary to draw more concrete conclusions on the effects of parasites on male mating behavior and condition in the western mosquitofish.

Parasite presence in females, however, did have a significant effect on male mating preferences in this study. In the male mate choice experiment, males mated significantly more with uninfected females. However, males did not also preferentially

associate with nonparasitized females, suggesting two possibilities. First, association time may not be a strong measure of male mating preferences in mosquitofish. Second, males may have been using association time with females as a means of inspecting the quality or phenotype of that female. Parasite index (ratio of parasite mass to female mass) was a marginally significant predictor of male mating behavior, accounting for about 17% of the variation, indicating that parasite size is important for male mating decisions. Although these results were marginal, I believe they are biologically significant because several males did not mate with parasitized females. Females infected with large nematodes have asymmetrically shaped abdomens, usually having a larger bulge on one side of their body. This change in female phenotype may be a mating cue used by male *G. affinis* to assess female quality. Further tests are needed to understand the specific mechanism by which this parasite affects male mate choice (e.g. vision vs. olfaction). However, these results clearly show that males prefer uninfected females, when given a choice, supporting the hypothesis that parasites mediate sexual selection via male mate choice *G. affinis*. Most studies to date on fish and other organisms have investigated parasite effects on female mating preferences (see Moller, 1990; Barber, 2002). Few studies have investigated the effects of parasites on male mate choice. To my knowledge, this is the first study to show that parasites can significantly influence male mating behavior in a species in which males of all sizes use coercive mating as their primary reproductive strategy.

In conclusion, parasites had significant effects on male, but not female, mate choice in *G. affinis*, a fish species exhibiting a coercive mating system. I expected parasites to influence mate choice in both sexes, especially if parasites altered behaviors

or phenotypes used as mating cues. Parasitic nematodes may differentially influence fitness of males and females. However, it appears that *E. ignotus* has a stronger influence on female than male fitness, since males preferred to mate with nonparasitized over parasitized females. If males selectively mate with nonparasitized females, which also may be larger and more fecund, this could lead to marked differences in mating strategies across populations of mosquitofish. This study provides new and exciting evidence that parasites may have strong influences on sexual selection via intersexual mechanisms (specifically male mate choice) in species where males typically do not show strong mating preferences for females. These results may generally apply to other species in which males use coercive mating as their primary means of obtaining reproductive success.

Acknowledgements

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Figure 1. Mean male mating behavior (a) and mean association time (b) with parasitized and nonparasitized females. Bars represent one standard error.

Figure 2. Male mating behavior as a function of female parasite index (MMB=2.07-0.0841PI; R^2=0.174, df=15, p=0.09).

Figure 3. Mean female condition (residuals) for parasitized and nonparasitized females. Bars represent one standard error.

Figure 4. Results of ANCOVA showing female dry mass (g) as a function of standard length (SL) for nonparasitized (closed circles) and parasitized (open circles) females.

Figure 1

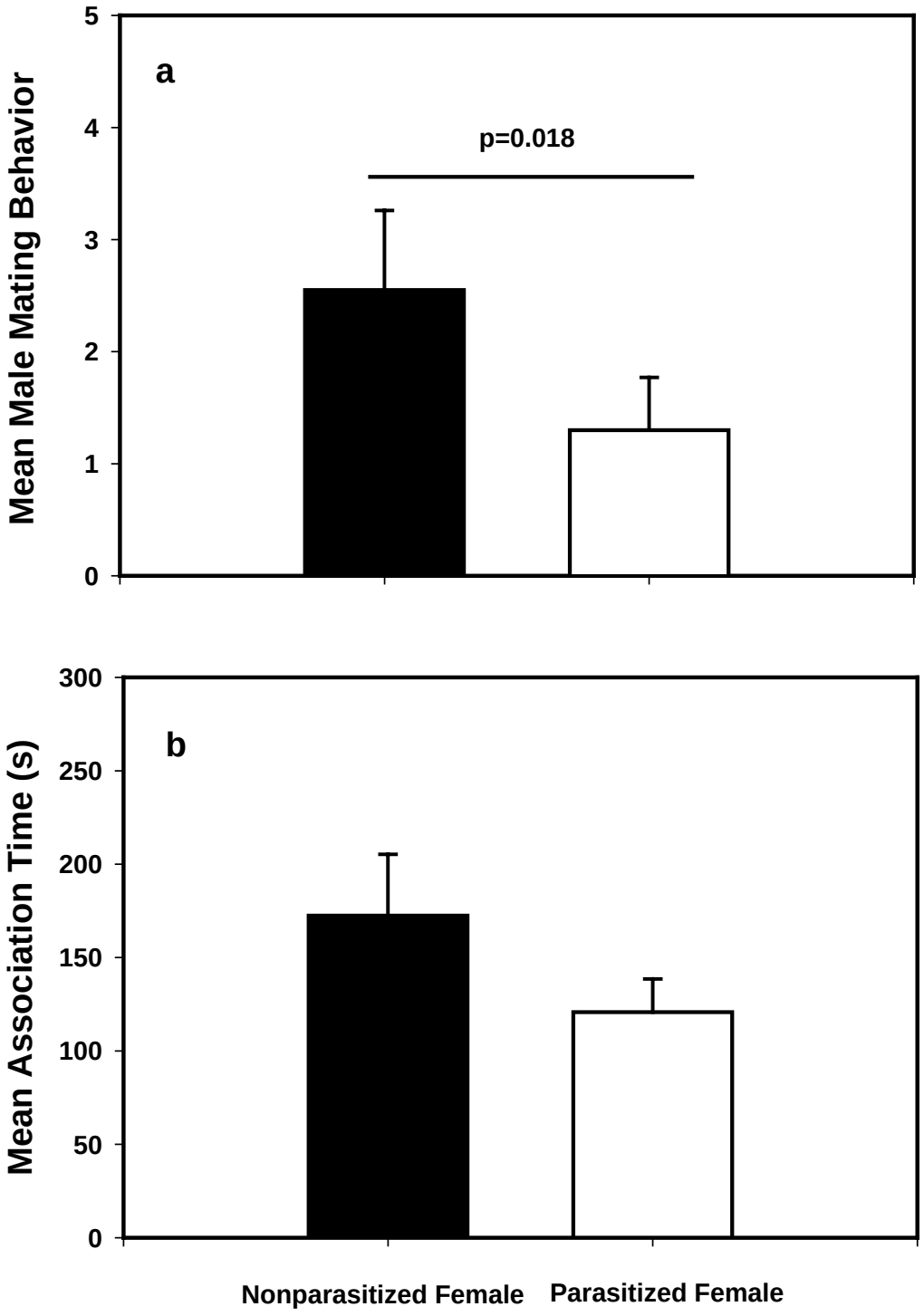

a
p=0.018
Mean Male Mating Behavior
5
4
3
2
1
0
b
Mean Association Time (s)
300
250
200
150
100
50
0
Nonparasitized Female
Parasitized Female

Figure 2

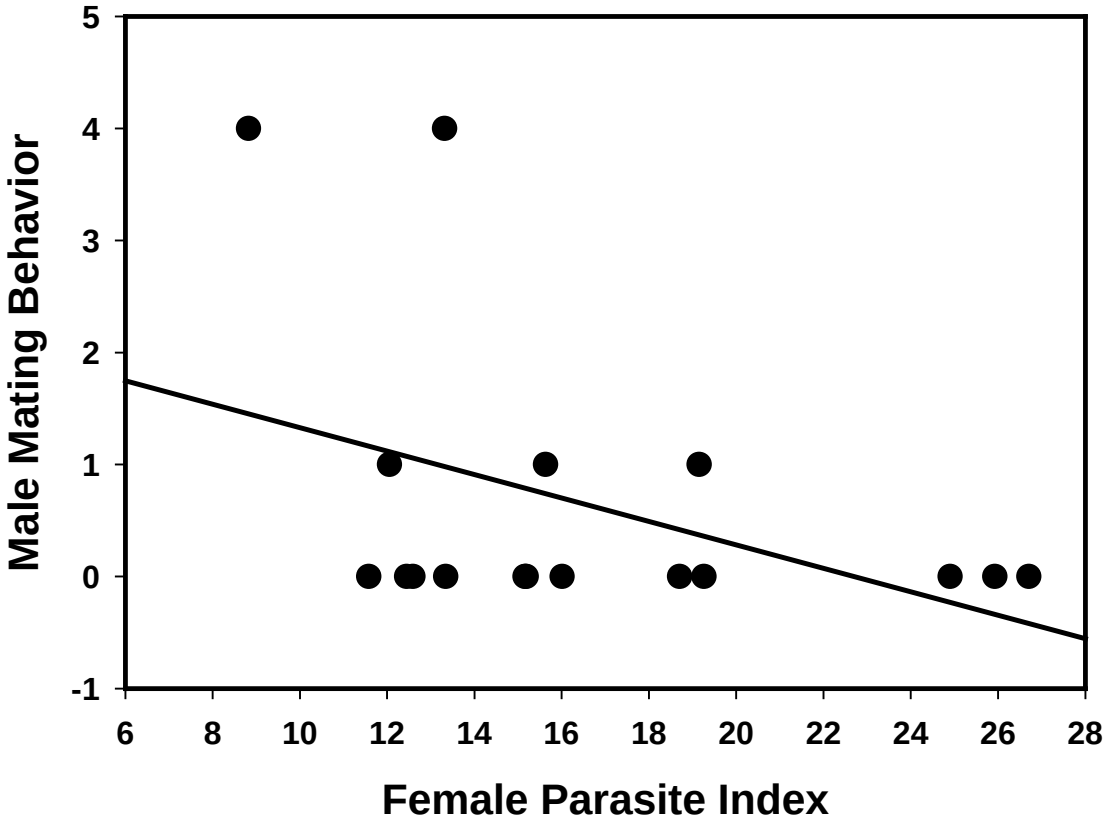

Male Mating Behavior
5
4
3
2
1
0
-1
6
8
10
12
14
16
18
20
22
24
26
28
Female Parasite Index

Figure 3

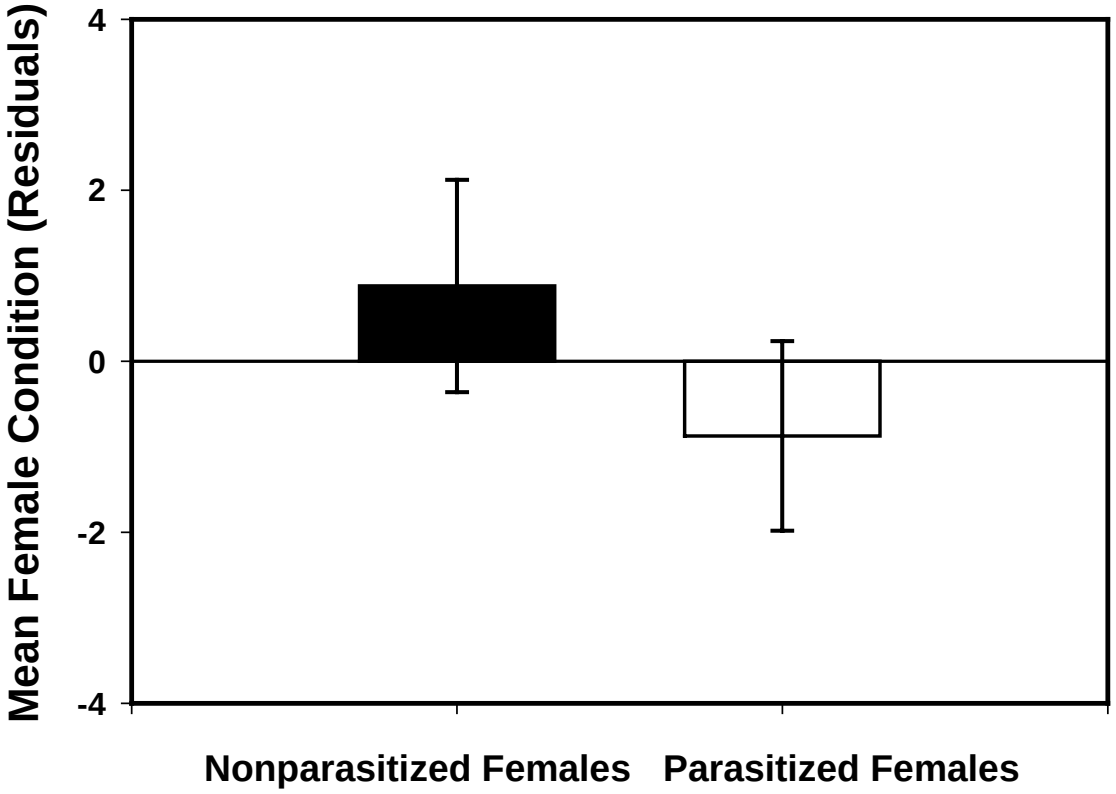
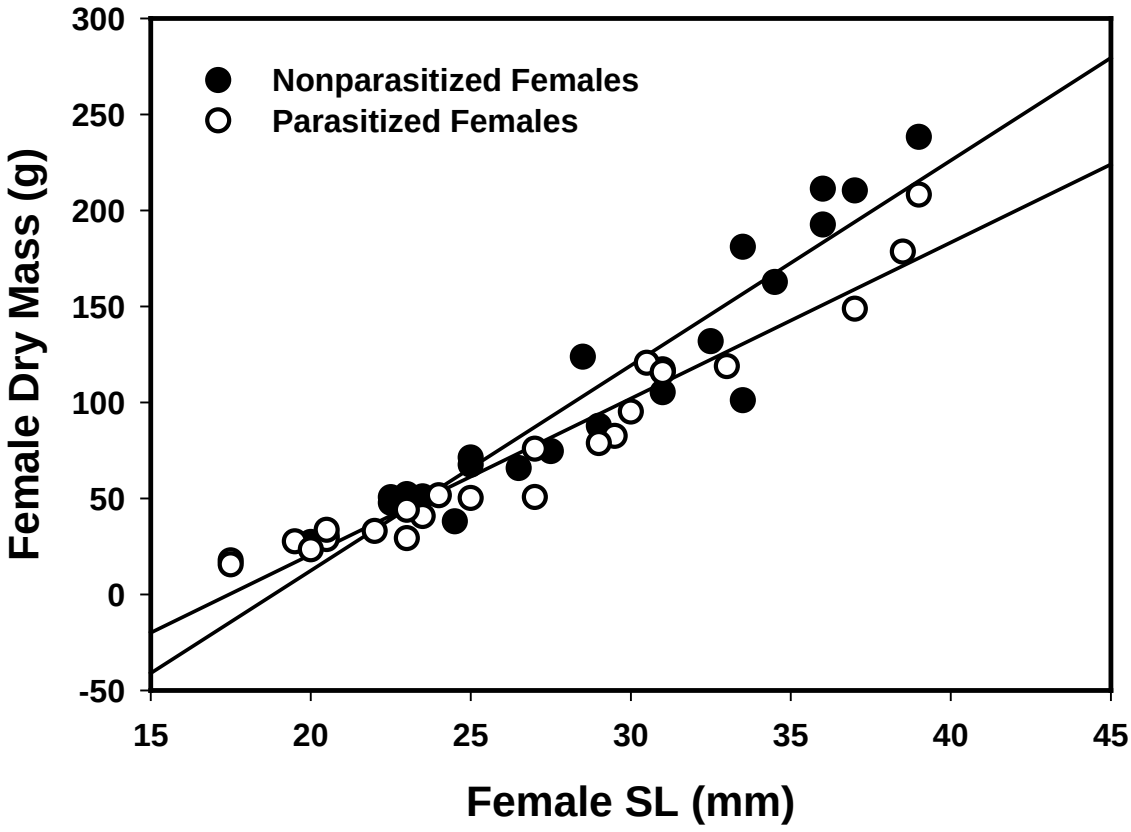

Mean Female Condition (Residuals)
4
2
0
-2
-4
Nonparasitized Females
Parasitized Females

Figure 4


Nonparasitized Females
Parasitized Females
Female Dry Mass (g)
300
250
200
150
100
50
0
-50
15
20
25
30
35
40
45
Female SL (mm)

CHAPTER III

Parasite effects on female reproduction in the western mosquitofish, *Gambusia affinis*

Raelynn Deaton

Abstract

Parasites are known to affect reproduction in many species, having both negative and positive influences on host reproduction. I tested the effects of infection by the gastro-intestinal parasitic nematode, *Eustrongylides ignotus* on female reproduction in the livebearing fish *Gambusia affinis*. In general, parasitized females had fewer developing broods than nonparasitized females. Of females carrying developing broods, brood size did not differ significantly between infected and uninfected females. Average parasite index was about 15%, and parasite index was negatively correlated with embryo number and female size. Parasitized females were in better condition than nonparasitized females, suggesting that infected females may store fat for growth or future reproduction. Results of ANCOVA showed a significant nematode-by-female size interaction, indicating that parasites affected size specific fecundity. This study shows that parasitic nematodes reduce fecundity in the western mosquitofish, and results suggest that parasites may have more profound affects on current than future reproductive investment.

Introduction

Parasitism is one of the most successful modes of life, as evident by the number of times it has evolved and by the diversity of parasitic species that exist (Poulin and Morand, 2000). As a result, parasite-host interactions have fascinated evolutionary biologists for decades. Because of their complex dynamics, host-parasite relationships have been studied from many perspectives, including coevolution (Solar & Solar, 2000; Webster and Davies, 2001; Garamszegi, 2006; Lohse et al., 2006, Servido & Hauber, 2006), behavioral shifts associated with infections (Barber, 2000 and Moore, 2002), parasite avoidance behaviors (Barber, 2000; Ezenwa, 2004; Apio et al., 2006), sexual selection (Moller 1990; Johnson, 1990; Mikinski & Bakker, 1990; Bronseth & Folstad, 1997) and reproductive life history (Sundberg, 1995, Heins et al., 1999, Heins et al. 2004). Parasites have been found to decrease host fitness directly through mortality, or indirectly through decreased fitness. Several studies have investigated the relationship between parasites and host reproduction (Heins et al., 1999, Heins & Baker, 2003), and results have reported both negative and positive effects on current and future reproductive investment. Although parasites do not typically kill their hosts, it is not unusual to detect reduction in fitness of organisms infected with parasites (Wiehn et al., 1997; Polak & Starmer, 1998). However, some studies have shown that parasites have no negative effects on host reproductive success (Shutler et al., 2004) and, in some cases, females increase their reproductive investment presumably to compensate for parasite infection (Cunningham & Lewis, 2006).

Reduced fitness is often manifested in reproductive life history characters, such as changes in offspring size and/or number (Fitzgerald et al., 1993; Sundberg, 1995) as well

as in other secondary fitness characters such as body condition (Hamilton & Zuk, 1982; Zuk et al., 1990; Fitzgerald et al., 1993; Polak and Starmer, 1998). Wiehn et al. (1997) showed that American kestrels infected with blood parasites suffered reduced fitness (via decreased reproductive success and body condition) due to increased susceptibility of reproductive individuals to infection by other parasites. Neuhaus (2003) found that removal of ectoparasites increased condition of lactating female ground squirrels, which lead to marked increases in reproductive success of treated females over an eight-year period. Some studies, however, have shown that individuals infected with parasites may invest more energy into current reproduction than into fat stores due to decreased life span (Polak & Starmer, 1998). Heins et al. (2004) found that body condition did not differ between infected and uninfected sticklebacks, but condition was negatively correlated with parasite index. Finally, some studies have shown parasitized individuals to be in better condition than their nonparasitized counterparts (Brooks, 2005), suggesting a more profound effect of parasites on current than on future reproduction.

Livebearing fishes are susceptible to many types of parasitic infections, including cestodes (Granath & Esch, 1983), black spot disease (Tobler et al. 2006), and nematodes (Coyner 1998; Coyner et al. 2001), among others. Some populations of *Gambusia* (mosquitofish) in Texas and Oklahoma are especially prone to infections by the gastro-parasitic nematode *Eustrongylides ignotus*. Mosquitofish serve as one of several intermediate hosts (in addition to other species of fish, amphibians, and reptiles; Coyner, 1998), while the primary host is a sediment-dwelling oligochaete and the terminal host a piscivorous bird (see Coyner, 1998). In the western mosquitofish, *G. affini*s, this parasite reaches up to 50% of female body mass (Deaton unpublished data), and consequently

may have significant negative effects on female reproductive success. Infection by this parasite has been associated with increased susceptibility to predation (Coyner et al., 2001), nutrient sequestering from the host (Marsh-Matthews, unpublished data), and decreased fecundity (Brooks, 2005) in *G. affinis*. Brooks (2005) investigated the relationship between resource availability and parasite presence in *G. affinis* and found that parasitized females suffered reduced fecundity (neonate mass and number), but were in better overall condition than nonparasitized females. However, a combination of low resources and parasite infection reduced host fitness by increasing mortality and reducing condition (Brooks, 2005). These findings suggest that this parasite has profound effects on mosquitofish reproductive life history, and these effects are complex, dynamic, and context dependent.

In this study, I examined the relationship between host reproduction and parasite infection from a natural population of *G. affinis* in Oklahoma, using embryo number as a measure of current reproductive investment, and body condition (based on soluble fats) as a measure of potential future reproductive investment. Based on previous research and personal observations, I expected parasites to affect female reproduction by reducing fecundity (egg/embryo number) and decreasing body condition (based on soluble fat). I also expected fecundity and body condition to decrease with parasite index.

Methods

I collected mosquitofish from a small pond in Norman, Oklahoma on 30 June 2002. This pond is habitat to a population of mosquitofish infected with *E. ignotus*. Fish were returned to a greenhouse laboratory and held for several weeks in a large community tank. During this time, fish were fed commercial flake food daily. I held fish

intentionally in the laboratory to allow for parasite growth, because *E. ignotus* grows throughout the reproductive season and reaches maximum size in late summer to early fall.

On 15 August 2002, I randomly chose thirty-one females from the community tanks, sacrificed them in MS-222, and preserved them in 10% formalin. I measured (to the nearest 0.5 mm standard length; SL) and dissected each female. The ovary was removed, embryos counted and staged (based on Meffe's six-stage scale, 1985), carcasses dried at 40ºC for 10 days, and weighed (to the nearest 0.001g).

Body condition was determined by weighing carcasses to the nearest 0.001 g, rinsing six times overnight (or longer) in petroleum ether to extract soluble nonstructural fats (Heulett et al. 1995; Trexler 1997), drying overnight at 40ºC, and reweighing. Body condition was quantified as the standardized residual from least squares linear regression of mass of somatic fat (calculated as pre-extraction mass minus post-extraction mass) on pre-extraction mass (Marsh-Matthews et al. 2005). For the seventeen parasitized females, parasites were removed, dried at 40ºC for 10 days, and weighed to calculate a parasite index (percent eviscerated parasite mass of percent eviscerated female carcass mass).

I tested for correlations between parasite index and body condition, female size (SL and dry mass), and fecundity (residuals of embryo number and female SL) and between condition and fecundity (embryo number; Table 1). Because fecundity is a function of female size, I used residuals of the regression of fecundity (embryo number) and size (SL) to correct for the correlation between the two variables (Brown & Prescott 1999). Parasite index was significantly correlated with female size (see Table 1);

therefore, I used regression analysis to test the predictive value of parasite index on female fecundity (embryo number). Because body condition was significantly correlated with female fecundity, I also used regression analysis to test for the predictive value of female condition on fecundity (embryo number).

I used a two-sample t-test to assess differences in body condition (residuals) between parasitized and nonparasitized females, and to test for differences in body condition between parasitized females with and without developing broods.

In this study, female fecundity was a function of both female standard length [EN=1.36(SL)-29.187, R^2=0.45, p<0.001] and dry mass [EN=0.206(DM)-3.88, R^2=0.52, p<0.001]. Therefore, I used ANCOVA to test for nematode effects on embryo number for the 20 females carrying developing broods (14 uninfected and 6 infected). Because results were similar for both SL and dry mass, I used female SL as a covariate. All statistical analyses were conducted in SPSS version 13.0 for Windows.

Results

Of the 31 females examined in this study, 17 were parasitized by *E. ignotus* and 14 were not, yielding a parasitism rate of 54.8%. Of the 17 infected females, 11 (or 65%) had no developing broods and only six females (or 35%) did have developing broods. Of the six infected females with developing broods, five females had stage six embryos and one female had stage five embryos. Average embryo number of the six infected females with developing broods was 17.5$\pm$4.37. Of the fourteen uninfected females, 11 (or 79 %) had developing broods. Eight females were carrying stage six embryos, two stage five embryos, and one stage one (or ripe eggs ready for fertilization). Three (or 21%) of

uninfected females were not carrying developing broods. Average embryo number for uninfected females carrying broods was 20.8±8.18.

Of the seventeen parasitized females, parasite index ranged from 1.7 to 34.7%, and averaged 15.1± 8.2 %. Parasite index significantly predicted embryo number (R^2=0.25, df=16, p=0.04; Figure 1), female SL (R^2=0.39, df=16, p<0.001; Figure 2a) and female dry mass (R^2=0.47, df=16, p<0.001; Figure 2b), indicating that smaller females have higher parasite indices than larger females.

Parasitized females were in better condition than nonparasitized females (two sample t-test, two-tailed, t=-2.34, df=30, p=0.02; Figure 3). There was no difference in body condition between parasitized females with and without developing broods (two sample t-test, two-tailed, t=0.12, df=16, p>0.05). Female condition accounted for a marginally significant amount of variation in embryo number (R^2=0.09, df=30, p=0.08). This relationship was negative, indicating that females in better condition had fewer developing embryos.

For the 20 females carrying developing broods (14 uninfected and six infected), analysis of covariance was used to test for nematode effects on embryo number. The whole model was significant (ANCOVA, R^2=0.563, $F_{3,19}$=6.863, p=0.003; Figure 4). In addition, there was a significant nematode by standard length interaction ($F_{1,19}$=4.431, p=0.05) and female SL was a significant covariate ($F_{1,19}$=14.291, p=0.002). There was a marginally significant nematode effect ($F_{1,19}$=3.506, p=0.08).

Discussion

This study shows a significant relationship between reproduction in females and presence of the gastro-parasitic nematode, *E. ignotus*, in a parasitized population of the

western mosquitofish, *G. affinis*. My results indicate that, in general, parasites have strong effects on both reproduction and condition of female mosquitofish. Rate of parasitism for the females in this study was about 55%, which is consistent with monthly field collections from the entire 2002 breeding (April – October, 2002). This parasite significantly affected female reproduction by reducing number of embryos in developing broods.

Parasite index was negatively correlated with both standard length and dry mass, indicating that smaller females had larger parasites (per unit size or mass) than larger females. These results suggest that smaller individuals may be more susceptible to parasite infections, or that parasites may retard growth. If smaller individuals are more susceptible to parasite infections, further studies are needed to determine causal mechanisms (i.e. ontogenetic foraging shifts). Female mosquitofish continue to grow throughout their lives (unlike males, which have determinant growth). It would not be surprising if *E. ignotus* slows female growth, if this parasite sequesters nutrients from its host. Because of the difference in male and female growth patterns in livebearing fish, it would be interesting also to test for correlations between male size and parasite index. Another explanation for smaller females having larger parasites could be that parasitized females had fewer developing broods than nonparastized females. In this case, parasites may have the opportunity to grow larger. Further research is needed to tease apart these alternative explanations.

While most of the nonparasitized females in this study were carrying developing broods (nearly 80%), most of the infected females did not have developing broods (nearly 65%). This could have significant negative consequences for female reproductive fitness

in parasitized populations of mosquitofish. It is unknown whether parasites caused females to delay investment into current reproduction, diminished energy stores due to nutrient theft, or if parasitized females had fewer mating opportunities with males. Results from male mate choice experiments suggest that males show mating preferences for uninfected females (Deaton, in prep). Further, males also prefer to mate with larger females (Deaton, in review). If smaller females are already at a mating disadvantage, and they are more likely to be parasitized, this may significantly reduce the overall reproductive success of parasitized females.

In addition, regression analysis showed that females in better condition had fewer eggs and or embryos, but this relationship was only marginally significant. Most nonparasitized females, on the other hand, had developing broods with several embryos. These females may have invested more energy into current reproduction than into fat stores. There was no significant correlation between female condition and parasite index, contrary to expectations. Females with larger parasites were not in poorer condition than females with smaller parasites. These results were surprising given that Marsh-Matthews (pers. com.) quantitatively showed uptake of nutrients by *E. ignotus* in mosquitofish hosts. However, stored soluble fat is a long term energy source; therefore, other, more immediate energy sources (as measures of condition), such as glycogen, lipids, or RNA to DNA ratios may yield different results.

Parasitized females were in better condition than nonparasitized females, which is also consistent with findings by Brooks (2005). Females in this study were housed in the laboratory, and fed high quality flake food daily for several weeks. Previous studies (Marsh-Matthews, Deaton, Brooks, unpublished data) showed that female mosquitofish

fed daily in the laboratory are in better overall condition than field-caught individuals. By giving all females equal rations of high quality food, the results of infected females being in better condition are even more convincing. Females carrying developing broods were in poorer condition (most of which were uninfected females), presumably because their current reproductive investment was greater than future investment of stored fats. Fat stores (body condition) are known to aid in surviving winter in guppies (Reznick and Braun, 1987) and to increase future reproduction in toads (Castellano et al., 2004). It appears that parasitized *G. affinis* females may trade-off current reproduction for survival and future reproduction. Parasitized females carrying broods, however, were not in better condition than parasitized female that were not carrying developing broods.

Results of analysis of covariance showed a significant nematode by size (SL) interaction, suggesting size specific effects of parasites on host fecundity. Parasitized females clearly had reduced brood sizes, and many infected females did not have developing broods. Uninfected females had larger brood sizes, and all uninfected females were carrying developing broods or large, ripe eggs ready for fertilization. Most infected females did not have developing broods at all, suggesting that parasite infection may decrease overall reproductive success by decreasing total number of broods in a given season.

These findings are consistent with those of Brooks (2005), who showed an increase in inter-brood intervals in parasitized female *G. affinis*. Reznick and Yang (1993) suggested that such an increase in brood intervals may be necessary for infected females to adequately yolk eggs. These findings are consistent with several other studies showing that parasitic infections reduce fecundity in fishes (Heins and Baker 2003;

Brooks 2005). For example, Heins et al. (1999) showed reduced clutch production in female sticklebacks infected with cestode larvae, but only during certain times in the breeding season. The present study was conducted at one point in time (during peak reproductive season), but it would not be surprising to find differences in levels of parasite effects on reproduction across the breeding season. This is because *E. ignotus* continues to grow throughout the summer, and does not reach maximum sizes until late in the reproductive season (October and November). In a related study, Brooks (2005) found that female mosquitofish infected with *E. ignotus* suffered decreased total brood mass (measured as neonate mass and neonate number). Thus, female mosquitofish infected with *E. ignotus* have significantly fewer embryos (as reported in this study), fewer neonates, and fewer broods in a given reproductive season than uninfected females.

In conclusion, results from this study suggest that parasites negatively affect female reproductive fitness (via reduced fecundity) by decreasing the overall number of developing broods, and by reducing number of eggs or developing embryos in parasitized females. Parasites had the opposite effect, however, on female condition. Parastitized females were in better condition based on soluble fat stores than nonparasitized females, suggesting that parasitized females may invest more energy into fat stores for future reproduction while uninfected females invest more heavily into current reproduction. These results are consistent with those of Brooks (2005) and other studies on the relationship between female reproduction and parasite infection. In mosquitofish, it appears that parasitized females may trade off current for future reproduction, which could be an adaptive strategy for coping with parasitic infections in populations where parasitic infections are prevalent.

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Table 1. Pearson correlation coefficients for parasite index (PI) and female standard length (SL), dry mass (DM), residuals of embryo number and SL (EN_SL), and body condition (BC), and for body condition (BC) and residuals of embryo number and SL (EN_SL).

	PI *n=17*	BC *n=31*
SL	-0.623**	--
DM	-0.688**	--
EN_SL	-0.218	0.373*
BC	-0.167	--

* indicates significance at $p<0.05$
** indicates significance at $p<0.01$

Figure 1. Parasite index as a function of female SL (a) and dry mass (b). Female SL [PI=131.0-4.07(SL), R^2=047, p<0.001] and female dry mass [PI=37.4-0.54(DM), R^2=039, p<0.001] significantly predicted parasite index.

Figure 2. Embryo number as a function of parasite index. Parasite index significantly predicted embryo number [EN=14.5-0.55(PI), R^2=0.25, p=0.04].

Figure 3. Mean female condition (residuals) of parasitized (black bar) versus nonparasitized females (white bar; two sample t-test, two-tailed, t=-2.34, df=30, p=0.02).

Figure 4. Analysis of covariance showing embryo number as a function of female size (SL). A significant interaction was detected for nematode and female SL ($F_{1,19}$=4.431, p=0.05). Female SL was a significant covariate ($F_{1,19}$=14.291, p=0.002), but there was only a marginally significant nematode effect ($F_{1,19}$=3.506, p=0.08). Parasitized females represented by filled circles and nonparasitized females represented by open circles.

Figure 1

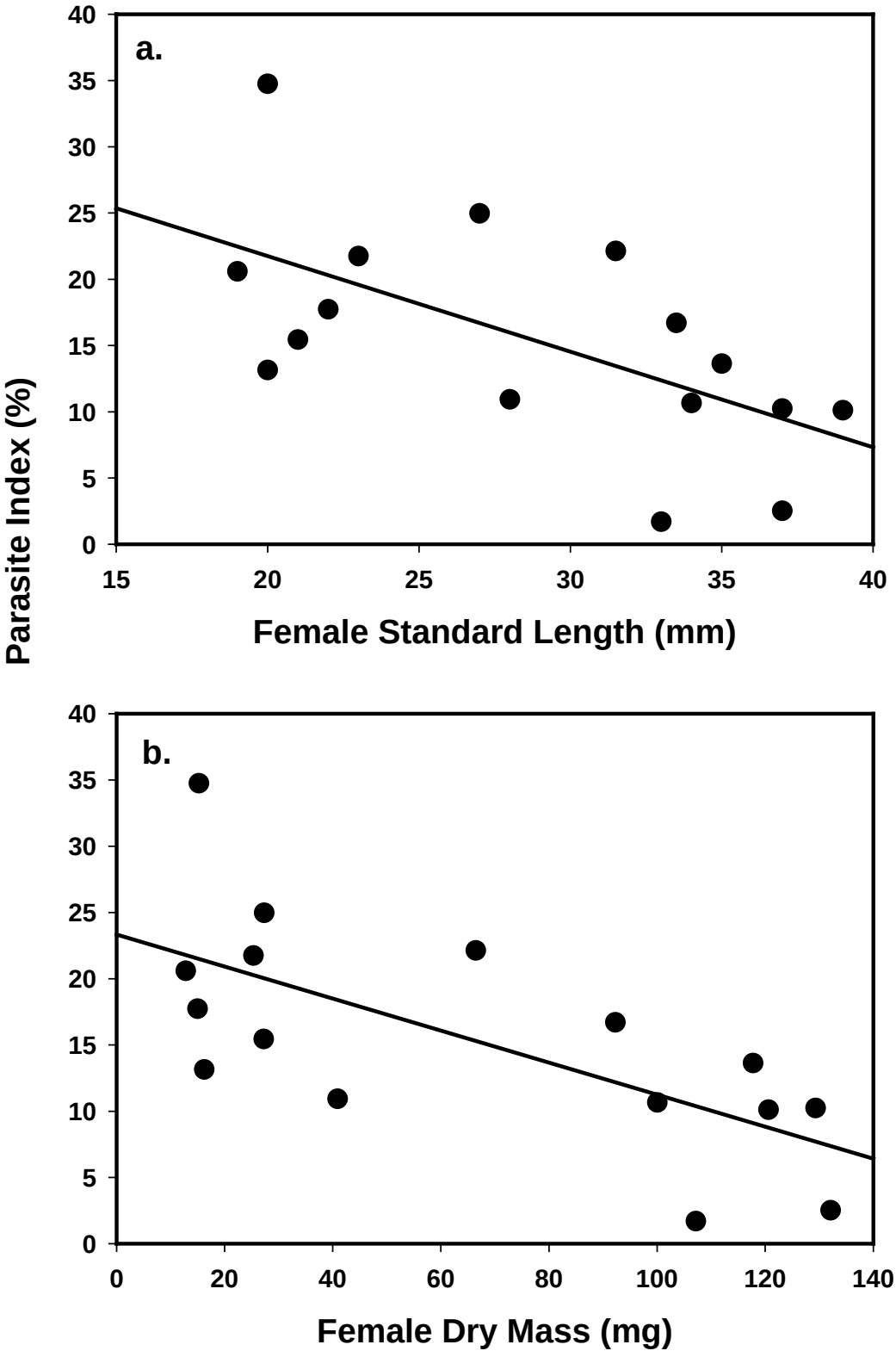

a.
Parasite Index (%)
Female Standard Length (mm)
b.
Female Dry Mass (mg)

Figure 2

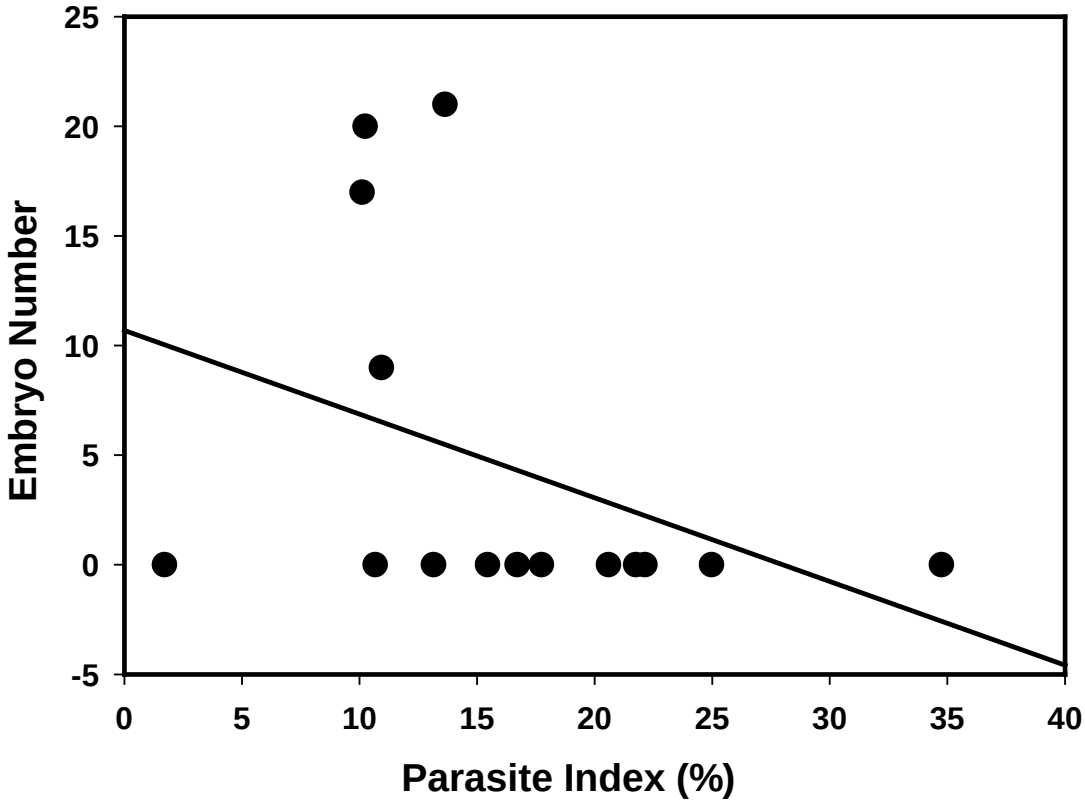

Embryo Number
25
20
15
10
5
0
-5
0
5
10
15
20
25
30
35
40
Parasite Index (%)

Figure 3

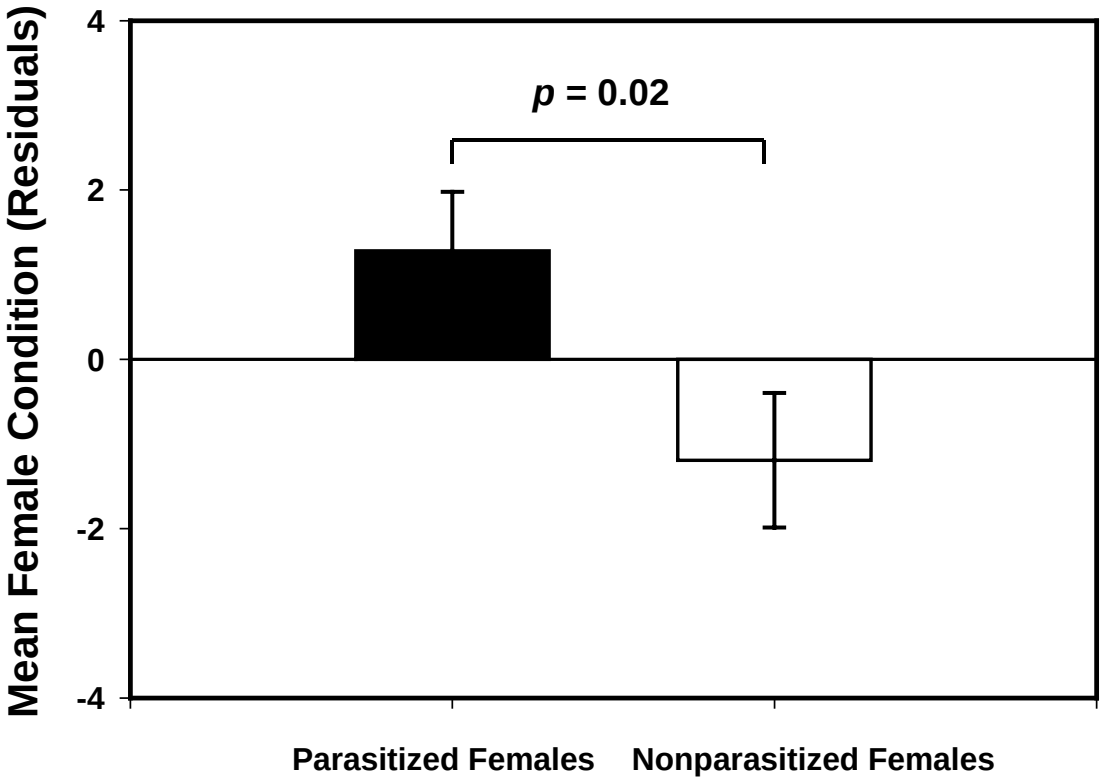
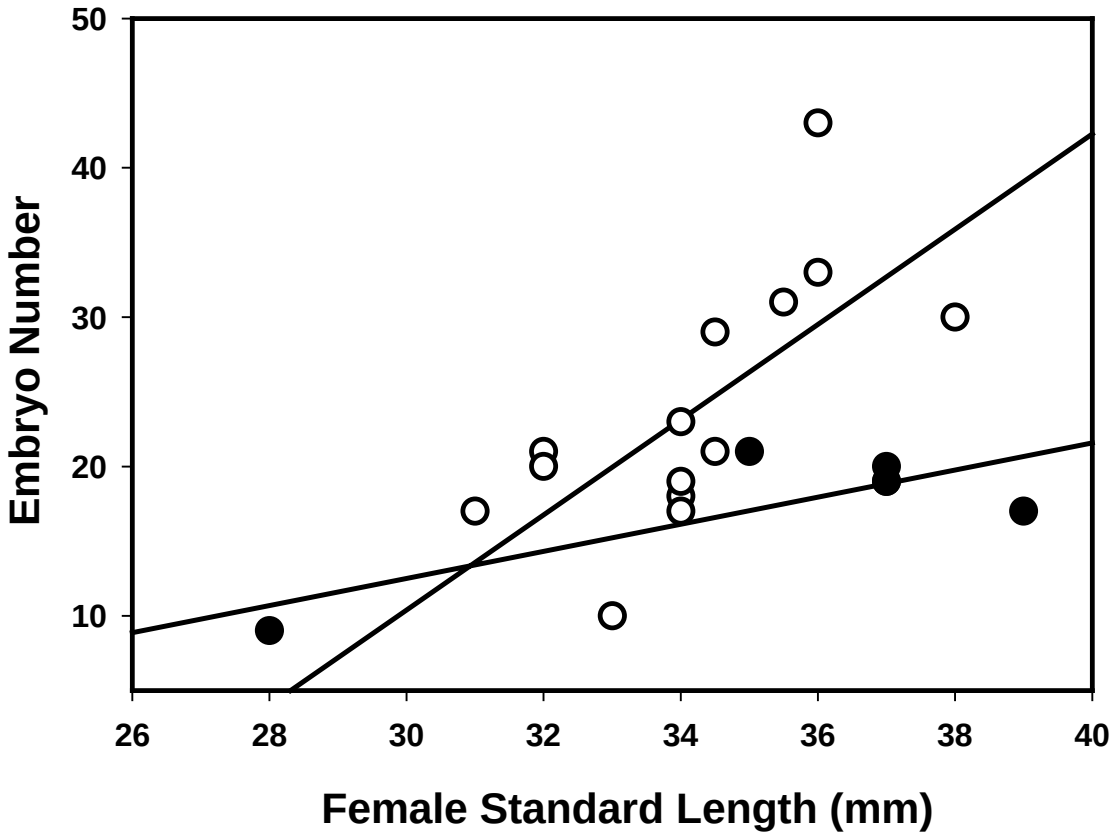

Mean Female Condition (Residuals)
4
2
0
-2
-4
p = 0.02
Parasitized Females
Nonparasitized Females

Figure 4


50
40
30
20
10
Embryo Number
26
28
30
32
34
36
38
40
Female Standard Length (mm)

CHAPTER IV

Male-male competition, size, and mating success in the western mosquitofish, *Gambusia affinis*

Raelynn Deaton

Summary

I tested the effects of male body size on male mating behavior and reproductive success in the western mosquitofish, *Gambusia affinis*. I hypothesized that male body size affects overall reproductive success. In two separate behavioral experiments (male-male competition and no-competition), I tested the predictions that (1) larger males will out-compete smaller males for mates and (2) small males would increase their number of mating attempts in the absence of a larger competitor. I estimated male mating success both indirectly (via behavioral experiments) and directly (using microsatellite DNA to assign parentage) and compared the two measures. Results from behavior experiments showed that, when in direct competition, large males were more aggressive and attempted more copulations than small males. In addition, paternity analyses illustrated that large males sired more offspring (at about a 2:1 ratio). I found no significant correlations between male body size (and other correlated traits) and mating success in the competition study, suggesting that relative male size influences male mating behavior but absolute male size does not. When competition was removed, small males mated at equal rates to larger males. Finally, indirect estimates of male reproductive success predicted results from parentage analyses, although this relationship was only marginally significant.

Introduction

Many factors are known to influence male mating behaviors, and ultimately, reproductive success, including dominance/aggression (Gozlan et al., 2003), body size (Kissner et al., 2005), parasites (Wiehn et al., 1997) and body condition (Kodric-Brown, 1989). Male body size has been shown to be an important component in both intrasexual competition and intersexual mate choice (Andersson, 1994), and is generally accepted as one of the most fundamental predictors of male reproductive success (Perrin, 1998; Wikelski, 2005). However, male size might also indirectly affect fitness via correlative associations with other traits (e.g. dominance status, aggression and condition; Haley et al., 1994; Teder, 2005; Candolin, 2005; Fisher et al., 2006). For example, larger males often have a competitive advantage over smaller males because they are more aggressive (Riesch et al., 2006) and thus, more likely to court females (Morris, 1991; Savalli & Fox 1999, but see Friedl & Klump, 2005). As a result, smaller males in some species often use alternative reproductive tactics such as sneaky or coercive (forced) mating (Bisazza & Marin, 1995; Gross, 1996; Pilastro et al., 1997).

The livebearing fishes in the family Poeciliidae show considerable variation in male size (Snelson, 1989), making them good candidates for studies of body size effects on mating success. For example, large sailfin molly males display an enlarged, brightly colored dorsal fin to attract females, while smaller males, lacking the sail-like fin, coerce females (Riesch et al., 2006). Also, in closely related guppies, larger, more colorful males court females, while smaller males use sneaky behaviors to obtain copulations (Houde, 1997). Traditionally, these alternative mating strategies by small males were viewed as less effective (Pilastro et al., 1997); however, alternative strategies recently

have been suggested as important mechanisms for the maintenance of small male body size relative to females in species with extreme size polymorphism (Bisazza & Pilastro, 1997). In the one sided livebearer, for example, the coercive strategy of small males gives them a mating advantage, possibly explaining the coexistence of small and large males in natural populations (Bisazza et al., 2000).

The livebearing mosquitofishes (Genus *Gambusia*) have pronounced male size polymorphism (Bisazza & Marin 1991, 1995; Zulian et al., 1995; Campton & Gall, 1988) where males have determinant growth (Snelson, 1989), maturing between 11-24 mm standard length (SL; Campton & Gall, 1988). The mosquitofish mating system is largely male driven, based on male coercion with no courting of females (Bisazza & Pilastro, 1997). Moreover, there is little evidence for female choice (Bisazza & Marin, 1991, 1995 but see Hughes 1985; McPeek, 1992; Gould et al., 1999; Bisazza et al., 2001), suggesting that intrasexual competition may be an important mechanism for the maintenance of male size variation. Little is known regarding the genetic basis for male size polymorphism in mosquitofish, but Campton and Gall (1988) showed a heritable component to male body size in *G. affinis* using quantitative genetics. In the related swordtails (and other livebearers), male size has been linked to variation in the pituitary locus on the Y-chromosome (commonly referred to as the "P gene"; Zimmerer & Kallman, 1989; Ryan et al., 1992). There is also a known social component to male size at maturity, (Campton & Gall, 1988; Snelson, 1989), further complicating the determinants of male body size in mosquitofish.

Hughes (1985) tested the effects of male body size in the western mosquitofish and found that large males outcompete smaller males for matings when in direct

competition. He estimated male mating success using an indirect, behavioral approach and showed that large male are more aggressive, and small males rely more on forced copulations than larger males. In this study, I used both behavioral methods (as in Hughes, 1985) and molecular tools to address the effects of body size (and other correlated traits) on male reproductive success in the western mosquitofish.

Based on findings by Hughes (1985), I hypothesized that body size influences overall mating success of male western mosquitofish, *G. affinis*. I predicted that large males would have higher reproductive success (number of offspring sired) than small males when in direct competition, presumably because they are more aggressive (as shown in the western mosquitofish; Hughes, 1985 and other livebearers; Bisazza & Marin, 1991; Bisazza et al., 2000) and thus, better competitors for mates. However, I also predicted that small males should gain at least a portion of matings and sire some offspring, since small males persist in natural populations, and Hughes (1985) suggested that small males may use alternative mating strategies. I also assessed male mating behavior in a noncompetitive situation, and predicted that small males would mate at rates equal to larger males when there is no larger competitor present. Finally, I compared indirect (via behavioral observations) and direct estimates of mating success (actual number of offspring sired) to assess the relationship between the two measures of mating success.

Materials and Methods

July 2005, I collected male mosquitofish from a small pond in Norman, Oklahoma. Fish were returned to a greenhouse laboratory and acclimated in two 340-l community tanks for two weeks. Because I could not collect enough small males from

the field, I also reared male offspring in the laboratory from pregnant females collected from the same pond in May 2005. All fish were fed commercial flake food daily.

Experiment I: male competition and paternity

Indirect measures of male mating success (behavioral observations)

To measure male mating behavior in a competitive setting, I visually categorized and sorted males by size into two groups (small and large), attempting to capture all natural size variation within each group. Small males ranged in size from 11 mm-18.5 mm SL and large males ranged from 19-26.5 mm SL (measured post-experiment). I placed males in each size class together in 10-l plastic boxes for one week prior to experiments. I used a free-swimming "choice" design (Houde, 1997) to estimate male mating success (number of mating attempts). Behavioral observations were conducted 05 August 2005 through 08 August 2005 and were made in early morning (between 0700 and 0930) or near dusk (1730 and 2030), as mosquitofish mating activity peaks during these times (C. Hubbs, personal communication). Previous studies have shown no difference in male mating between early morning and evening (Deaton, unpublished data); therefore, data from morning and evening samples were pooled.

For each behavioral trial, I randomly selected one male from each of the two size groups and one female. I attempted to vary the difference between the sizes of the males for each replicate. For each trial, I matched females for size (within 2-3 mm SL) and other phenotypic characteristics such as pigmentation, girth, and size of gravid spot (a dark pigment spot which appears in the abdominal region when females are gravid, Snelson, 1989) to minimize the effects of female phenotypes on male mating behaviors. Also, I used virgin females because female mosquitofish can store sperm for up to several

months (Constantz, 1989). I allowed fish to acclimate for 10 minutes in 20-liter experimental aquaria prior to data collection. All aquaria were lined with a standardized amount of gravel (approximately 2cm deep), emptied and rinsed thoroughly between trials.

Following the acclimation period, I conducted 5-min focal observations on each male. I recorded the number of copulation attempts by each male (measured as the number of times the male thrust his gonopodium toward the female's genital pore), and all aggressive behaviors toward the other male. Common male aggressive behaviors included chasing or lunging toward the other male, nipping, back arch and gonopodial display (Krotzer, 1990; Houde, 1997). Aggression was quantified as the sum of the recorded aggressive behaviors (see Hughes, 1985).

Direct measures of male mating success (paternity analysis)

Following behavioral trials, each experimental group (small and large male plus female; N=27) was placed in a 3.7-l plastic box with a mesh cover for several weeks. Fish were fed commercial flake food once daily (to excess) and checked for neonates two to three times daily. Females near parturition (those with extremely large gravid spots and a high width to length ratio) were isolated in breeding traps to prevent cannibalism (Hubbs, 1991). Once females started to give birth, males were removed from the box and placed in a separate container to prevent predation. After females completed parturition, neonates were collected and preserved in ethanol for DNA extractions. Most females were still gravid several weeks into the experiment. Only four females had given birth to live neonates and several females gave birth to dead neonates or aborted their broods prematurely. Therefore, on 10 September 2005, I euthanized females by stunning them

in ice water and immediately preserving them in ethanol for DNA analyses. I also stunned the males in ice water, quickly clipped a portion of the caudal fin for genetic analyses, and immediately preserved the remainder of the carcass in 5% formalin.

I dissected fertilized embryos from each female, and extracted DNA using Chelex (R) 100 resin (Burkhart et al., 2002) from all neonates and embryos per female, females (using a portion of the musculature tissue at the caudal peduncle), and potential fathers (using ethanol-preserved fin clips). Nineteen of the 27 females had developing broods, most of which were late stage embryos (stage 4 to stage 6; based on Meffe, 1985). I did not process the eight nongravid females for genetic analyses. Of the nineteen females processes for genetic analyses, I was able to determine paternity of offspring from nine females.

After conducting a preliminary experiment to test for variation in microsatellite loci developed for *G. affinis* (Spencer et al., 1999), I used the two most variable loci for my population (*Gaf 2* and *Gaf 4*) to assess paternity of offspring (see Table 1). Assigning parentage allows for a direct measure of mating success by estimating the number of offspring sired by each male. I used standard polymerase chain reaction (PCR) with fluorescent labeled primers, to amplify microsatellite markers for paternity analyses. Genetic analyses were conducted in the Systematics Laboratory at the Oklahoma Biological Survey and OU Department of Zoology Multi-User Molecular Laboratory using Applied Biosystems 3130XL Genetic Analyzer to generate microsatellites. Genotypes for each individual were determined with the aid of GeneMapper version 3.7 (Applied Biosystems).

Other male measurements

I measured male standard length and gonopodium length to the nearest 0.5 mm. Males were dissected, testes and liver removed, and dried along with carcasses for 10 days at 40º C. To assess body condition, carcasses and livers were weighed to the nearest 0.001 g, rinsed six times overnight (or longer) in petroleum ether to extract soluble nonstructural fats (Heulett et al., 1995; Trexler, 1997), dried overnight at 40ºC, and reweighed. Body condition was quantified as the standardized residual from least squares linear regression of mass of somatic fat/liver fat (calculated as pre-extraction mass minus post-extraction mass) on pre-extraction mass (Marsh-Matthews et al., 2005).

Field paternity

To validate that multiple paternity occurs in the natural habitat, I collected ten gravid females in May 2005. I returned the fish to a greenhouse laboratory facility and isolated each female in a 3.7-l plastic box with a mesh cover. Females were checked three-four times daily for neonates. As neonates were detected, the female was placed in a breeding trap to prevent cannibalism (Hubbs, 1991). After giving birth to the entire brood, the female and all neonates were euthanized by stunning in ice water, followed by immediate preservation in ethanol for DNA analyses. I used a commercial kit (DNeasy from Qiagen) to isolate DNA from the females (using caudal peduncle musculature) and twenty randomly selected neonates and/or embryos per brood. I used the most variable microsatellite locus for my study population (*Gaf 4*) to assess paternity following the same methods described above. I used direct counts of offspring and maternal alleles to determine the minimal number of paternal genotypes per brood.

Experiment II: male mating in a non-competitive situation

To test for effects of size on male mating behavior when male-male competition was removed, I followed the same methods as described for Experiment 1, except here I tested one male (randomly chosen from a group of males varying in size) with one non-virgin female using a "forced choice" (or "no choice"), free-swimming experimental design (Shackelton et al., 2005). This design has the advantage of testing male mating behaviors while eliminating the confounding effects of male-male interactions, but perhaps the disadvantage of being a less realistic mating scenario for mosquitofish. Following the experiment, I euthanized males by stunning them in ice water, followed by preservation in 5% formalin. Males were then measured to the nearest 0.05 mm SL. Females were returned to their community tanks and were not sacrificed following the experiment because they were used in subsequent studies.

Statistical approach

I tested for normality of data using Kolmogorov-Smirnov Test (Sokal & Rohlf, 1997). All data were normally distributed with the exception of male aggressive behaviors. Therefore, I performed both nonparametric and parametric statistics to test for differences between pairs in both experiments (competition and no competition). Results from nonparametric (Wilcoxon Signed-Rank Test) and its parametric counterpart (paired t-test) yielded very similar results. Therefore, I present results from parametric statistics in this report. I report two-tailed results for all analyses.

Because of the non-independence of data in the competition study (due to two males per replicate), I used the difference between the two males for the dependent variable (male mating attempts) and independent variables (male body size, testes mass,

gonopodial length, and condition). In addition, because male body size and other male characteristics measured were highly correlated (Pearson correlation, $r \geq 0.67$, $p<0.01$, $n=18$), I used residuals of the regressions of each male trait on male body size (SL) as independent variables (Brown & Prescott, 1999). Therefore, male body size (SL), residuals of the regression of gonopodial length on SL (GL_SL), somatic body condition on SL (SBC_SL), liver body condition on SL (LBC_SL), and testes mass on SL (TM_SL) were predictor variables for male mating attempts (measured as the number of gonopodial thrusts).

I used linear regression to compare indirect (number of mating attempts) and direct (number of offspring sired) measures of male mating success, using one randomly selected male from each pair to eliminate the confounding effects of non-independence of males in each trial. All statistical analyses were performed in SPSS version 13.0.

Results

Experiment I: male competition and paternity

Large males were twice as aggressive (paired t-test, $t=-3.28$, $df=20$, $p<0.01$; Figure 1a), attempted twice as many matings (paired t-test, $t=-1.83$, $df=20$, $p=0.08$; Figure 1b) and sired two times the number of offspring as small males (paired t-test, $t=2.3$, $df=8$, $p<0.01$, Figure 2a), although the statistical relationship between male size and mating behavior was marginally significant. The difference in male body size did not predict number of mating attempts, aggression, or number of offspring sired for large or small males ($p>0.05$), and the additional male characteristics measured (while controlling for body size using residual analysis) did not affect male mating ($R^2=0.078$, $n=18$, $p=0.889$). Therefore, relative male size is important in that larger males out-

compete smaller males when in competition; however, variation in male size alone does not predict mating success.

Of the nine broods for which paternity was assigned with high levels of certainty (see Table 1), eight of the nine broods showed mixed parentage and only one brood was sired by a single male (the larger male; Table 2). In all of the mixed broods, the large males sired about four times the number of the offspring as small males (Table 2). Within the nine broods for which paternity was assigned, large males attempted about three times the matings as small males (paired t-test, t=2.3, df=8, p=0.01, Figure 2b). The number of mating attempts predicted the number of offspring sired (R^2=0.41, N=9, p=0.06; Figure 3), but with marginal significance.

Field paternity

In the wild population, I found evidence for mixed paternity in four of ten broods, with at least two fathers per brood (Table 3). The other six broods were not variable at the *Gaf4* microsatellite locus, and thus, mixed paternity could not be confirmed. This is a conservative estimate because it is based on allele counts, yielding minimal numbers of paternal genotypes per brood.

Experiment II: male mating in a non-competitive situation

There was no significant difference between number of mating attempts between large and small males (two-sample t-test, t=2.1, df=18, p=0.96), averaging 3.1 versus 3.0 mating attempts respectively.

Discussion

In this study, I tested the hypothesis that male body size influences overall reproductive success in the western mosquitofish, *G. affinis*. I measured male mating

success both indirectly (via behavioral sampling of male mating attempts, see also Hughes, 1985) and directly (using microsatellite paternity analysis) in a competitive and non-competitive situation. In the male competition experiment, my results generally supported the prediction that large males out-compete smaller males for mates. These results were not surprising, considering that Hughes (1985) also reported similar results of *G. affinis*. I also found that larger males tend to attempt more copulations than smaller males (although this relationship was marginal), and are more aggressive than small males. Similar results have been reported the western mosquitofish (Hughes, 1985) as well as many other livebearing fishes (Houde, 1997), including the closely related eastern mosquitofish, *G. holbrooki* (Bisazza & Marin, 1995).

In this study, I examined the degree to which large males out-compete smaller males in a male-male competition experiment. Although I predicted that large males would have a competitive advantage (presumably because they are more aggressive), I also predicted that small males would acquire some matings, even in direct competition with larger males. I tested this prediction using microsatellite DNA to determine the actual number of offspring sired by males. Smaller males attempted about half the number of matings and sired almost half of the number of offspring as larger males. In all but one brood, small males fathered at least a quarter of the offspring as the larger male. My results show that even though large males have a competitive advantage over small males, they do not completely exclude smaller males from mating with females. Hughes (1985) also showed a large male competitive advantage in *G. affinis*, but only when presented with non-virgin, male-deprived females. In this study, I used virgin females, which were also male deprived, and did not compare male mating behavior

between virgin and non-virgin females. Because female mosquitofish store sperm (Constantz, 1989), it was necessary to use virgins to accurately determine reproductive success using genetic analyses.

In a non-competitive situation, Hughes (1985) found that small males were more likely to use forced inseminations, while large males were more likely to court, and suggested size correlated mating differences by males. It is possible that small males use alternative reproductive tactics, such as sneaky copulations to gain matings during competitive and/or non-competitive situations, which has been suggested for other livebearing fishes (Houde, 1997). However, this has not been directly tested for *G. affinis*. In the no competition experiment, I found no difference between the number of mating attempts of large and small males. These results differ somewhat from those found in the one-sided livebearer, where small males mated at higher frequencies than large males when no larger competitors were present (Bisazza et al., 2000). I found no evidence for a small male mating advantage in *G. affinis* in this study, but further tests are needed to draw definitive conclusions.

In many species, male body size can influence mating success either directly (Savalli & Fox, 1999), or indirectly (Wikelski, 2005). In this study, I used a multiple regression to test for predictive effects of male size and other correlated traits that may also influence mating success. I found that variation in male body size (or any other trait correlated with body size) did not predict male mating behavior. However, large males attempt more matings and obtain higher reproductive success than small males when in a competitive situation.

Most studies on mating behavior in livebearing fishes assume that indirect measures of mating success accurately predict number of offspring sired (McPeek, 1992; Pilastro et al., 1997; Bisazza et al., 2000, 2001). However, the extent to which indirect measures can be used to predict actual mating success (using paternity measures) has only been tested in one livebearing fish, *Limia perugiae* (Schartl et al., 1993), a species with strong female choice for colorful males. Based on nine families tested, number of mating attempts by males marginally predicted the number of offspring sired, but accounted for a large amount of variation in parentage (41%). This suggests that with large enough sample sizes, indirect measures of mating success should be strongly predictive of actual fertilization success. I believe these results have strong implications for behavioral studies of livebearing fishes (especially the mosquitofishes), as they show that indirect measures of reproductive success can be used to predict the actual number of offspring sired by males. These findings are important because molecular and genetic techniques are expensive and time consuming. Thus, if indirect measures of mating success can be used as a surrogate measure for actual reproductive success, then researchers can save time and money by conducting behavioral studies to predict male reproductive success.

Most researchers studying mosquitofishes assume that multiple paternity occurs in natural populations. Multiple paternity in a wild population of the closely related eastern mosquitofish (*G. holbrooki*) has been reported by Zane et al. (1999), and Green and Brown (1991) reported mixed paternity in the western mosquitofish using electrophoresis techniques. This is the first published report of the actual measures (number of fathers per brood) of multiple paternity in a wild population of *G. affinis*.

Unfortunately, my study population was not variable at several microsatellite loci, possibly due to having undergone severe population bottlenecks. There was only one microsatellite locus with enough genetic variation to determine minimum numbers of fathers per brood. Also, I did not know the size of the fathers assigned from the field paternity study. It would be interesting to determine if large males also have an advantage in the wild, when sex ratios, competition, and density are dynamic. Nonetheless, this study confirms that multiple paternity occurs in both the laboratory and a natural population of the western mosquitofish. This, coupled with the verification that male mating success can be predicted by indirect, behavioral studies, provides valuable information for behavioral ecologists studying the mating system of livebearing fishes, especially those exhibiting a coercive mating system.

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Table 1. Maternal and paternal genotypes, and offspring alleles for the nine broods for which paternity was assigned with high levels of certainty (at least 94% of the brood) in the male-male competition experiment. Genotypes presented are from the microsatellite locus used to assign paternity for that brood (also shown). The number of offspring scored per brood (# scored) divided by the total number of offspring in that brood (total), yielding a percent of the brood that was assigned paternity (% brood scored) is also presented.

Brood #	Maternal genotype	Paternal genotypes (large:small)	Offspring alleles	Locus	# scored/total (% brood scored)
2	231/231	231/231(lg) 233/233(sm)	231 233	*Gaf4*	16/17(94%)
4	189/227	189/197(lg) 233/233(sm)	189 197 227 233	*Gaf4*	8/8(100%)
5	276/276	259/276(lg) 257/257(sm)	257 259 276	*Gaf5*	24/25(96%)
7	189/231	197/233(lg) 231/231(sm)	189 197 231 233	*Gaf4*	19/19(100%)
8	189/197	189/197(lg) 231/231(sm)	189 197 231	*Gaf4*	28/29(97%)
13	197/231	197/233(lg) 231/231(sm)	197 231 233	*Gaf4*	15/15(100%)
17	233/233	189/233(lg) 197/233(sm)	189 231 233	*Gaf4*	9/9(100%)
19	257/276	259/259(lg) 276/276(sm)	257 259 276	*Gaf5*	20/20(100%)
26	259/276	276/276(lg) 259/259(sm)	257 259 276	*Gaf5*	13/13(100%)

Table 2. Comparison of male mating success (number of offspring sired) from male-male competition experiment. Brood number (female), male size (measured as standard length (SL) the nearest 0.5 millimeter (mm), microsatellite locus used to assign paternity, number of offspring sired by each male (large vs. small), and approximate ratio of large male to small male mating success are shown. Only those broods in which paternity for at least 94% of all offspring could be assigned are reported.

Brood # (Female)	Male SL(mm) (Large/Small)	Microsatellite (Locus)	# (%) Offspring Sired (Large/Small)	Approx. Ratio (Large:Small)
2	24.5/12.0	*Gaf4*	12(0.75)/4(0.25)	3:1
4	22.0/17.0	*Gaf4*	5(0.63)/3(0.37)	3:2
5	23.0/14.5	*Gaf5*	15(0.63)/9(0.37)	3:1
7	25.0/19.5	*Gaf4*	11(0.58)/8(0.42)	3:2
8	22.0/18.0	*Gaf4*	23(0.82)/5(0.18)	4:1
13	22.0/20.0	*Gaf4*	9(0.6)/6(0.4)	3:2
17	22.0/20.0	*Gaf4*	7(0.78)/2(0.22)	4:1
19	23.5/15.0	*Gaf5*	10(0.5)/10(0.5)	1:1
26	24.0/16.0	*Gaf5*	13(1.0)/0(0)	1:0

Table 3. Evidence of mixed paternity in four field caught females (out of 10) from my study site (a small pond in Norman, Oklahoma). Brood number (*n* underneath represents number of offspring successfully genotyped per brood), maternal genotype (mat), all alleles present in offspring for each brood (maternal and paternal; mat/pat), offspring genotypes, paternal genotypes (pat), and minimum number of fathers per brood are reported.

Brood # *n*	Genotype (mat)	Alleles/Brood (mat/pat)	Genotypes (offspring)	Paternal genotypes	Fathers/Brood (minimum #)
F3-05	230/230	186	230/230	4	2
n=16		196	230/232		
		230	186/230		
		232	196/230		
F4-05	230/230	230	230/230	4	2
n=16		232	230/232		
		186	186/230		
		196	196/230		
F6-05	230/230	230	230/230	4	2
n=14		232	230/232		
		186	186/230		
		196	196/230		
F7-04	196/232	196	196/196	3	2
n=20		189	232/232		
		232	186/232		

Figure 1. Comparison of mean number of aggressive behaviors (a) between large and small males (paired t-test, t=-3.28, df=20, two-tailed, p<0.01) and mean number of mating attempts [(b); measured as number of gonopodial thrusts)] between large and small males in the male-male competition experiment (paired t-test, t=-1.83, df=20, two-tailed p=0.08). Error bars represent one standard error.

Figure 2. Comparison of paternity [(a); measured as mean number of offspring sired) between large and small males in the male-male competition experiment for nine broods (paired t-test, t=2.3, df=8, two-tailed, p=0.01) and mean number of mating attempts [(b); measured as number of gonopodial thrusts)] between large and small males for the nine broods for which I was able to assign parentage (paired t-test, t=3.35, df=8, two-tailed, p=0.005). Error bars represent one standard error.

Figure 3. Male mating success (measured as the number of offspring sired) as a function of number of mating attempts (measured as the number of gonopodial thrusts; MMS=2.18GT+ 4.39, R^2=0.41, N=9, p=0.06).

Figure 1

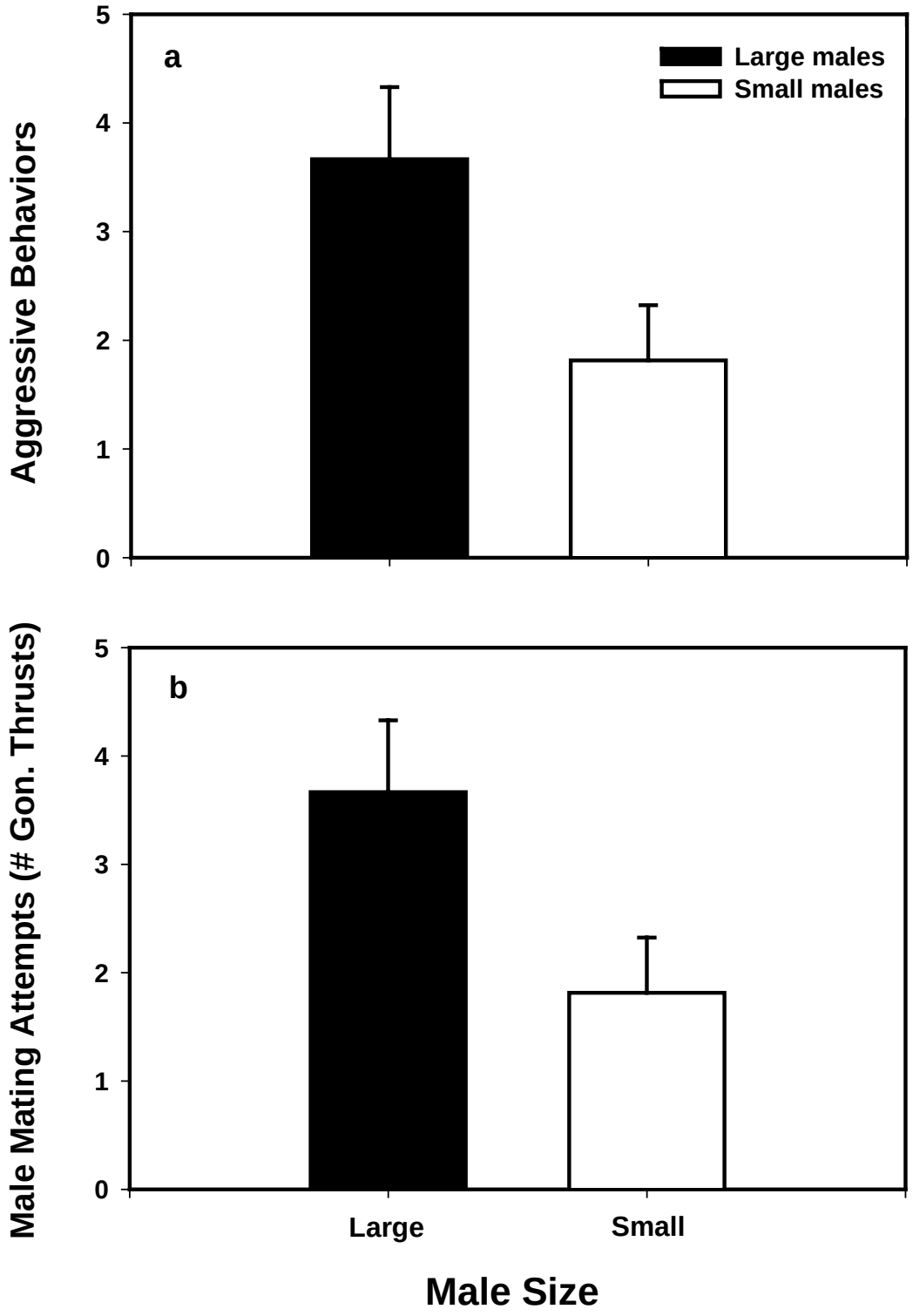

a
Large males
Small males
Aggressive Behaviors
0
1
2
3
4
5
b
Male Mating Attempts (# Gon. Thrusts)
Large
Small
Male Size

Figure 2

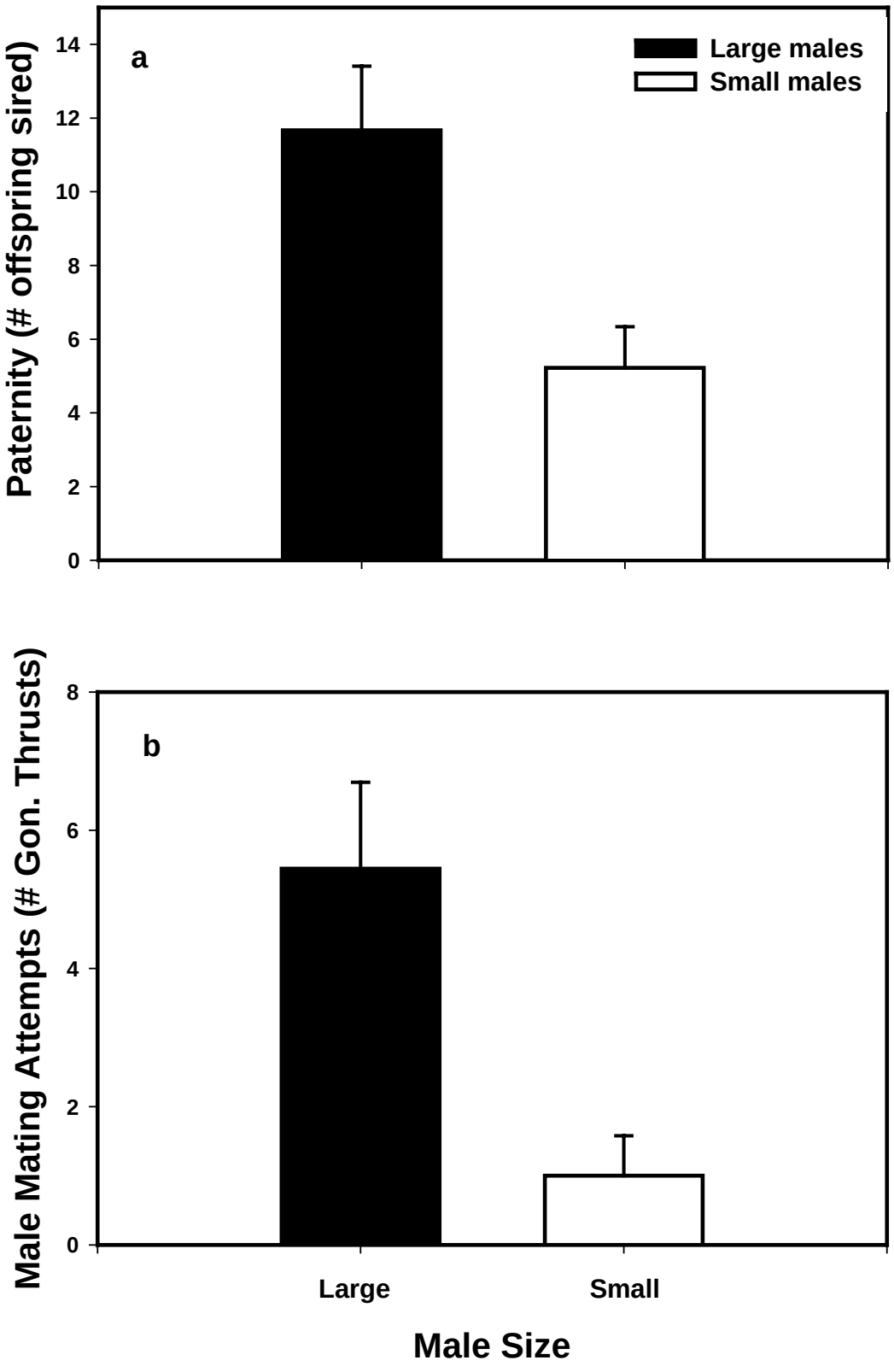
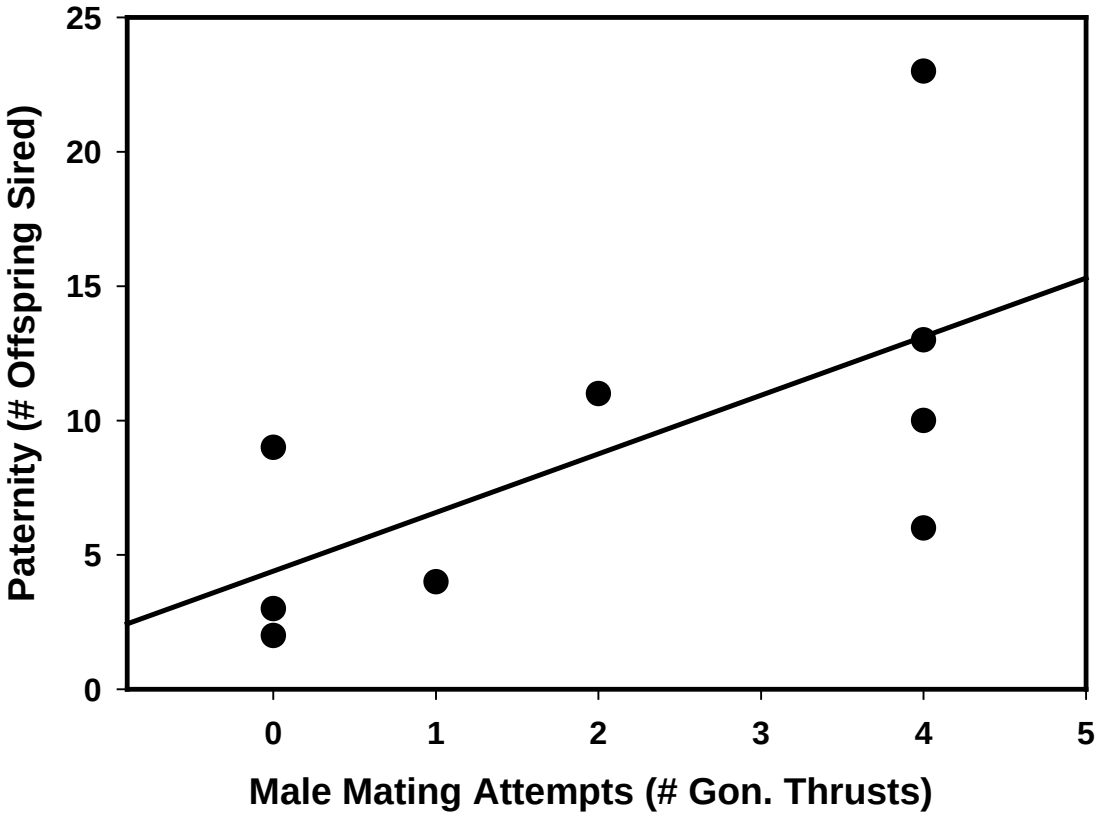

a
Large males
Small males
Paternity (# offspring sired)
0
2
4
6
8
10
12
14
b
Male Mating Attempts (# Gon. Thrusts)
0
2
4
6
8
Large
Small
Male Size

Figure 3


Paternity (# Offspring Sired)
25
20
15
10
5
0
0
1
2
3
4
5
Male Mating Attempts (# Gon. Thrusts)